EXHIBIT AL

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Watanabe et al.

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(45) **Date of Patent: *Jun. 8, 2021**

(54) **ANTISENSE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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Related U.S. Application Data

(63) Continuation of application No. 17/126,366, filed on Dec. 18, 2020, which is a continuation of application No. 16/408,529, filed on May 10, 2019, now Pat. No. 10,870,676, which is a continuation of application No. 15/619,996, filed on Jun. 12, 2017, now Pat. No. 10,329,319, which is a continuation of application No. 14/615,504, filed on Feb. 6, 2015, now Pat. No. 9,708,361, which is a continuation of application No. 13/819,520, filed as application No. PCT/JP2011/070318 on Aug. 31, 2011, now Pat. No. 9,079,934.

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(51) **Int. Cl.**

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C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
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C12Q 1/68	(2018.01)

(52) **U.S. Cl.**
CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — Sean McGarry

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(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

5 Claims, 19 Drawing Sheets

Specification includes a Sequence Listing.

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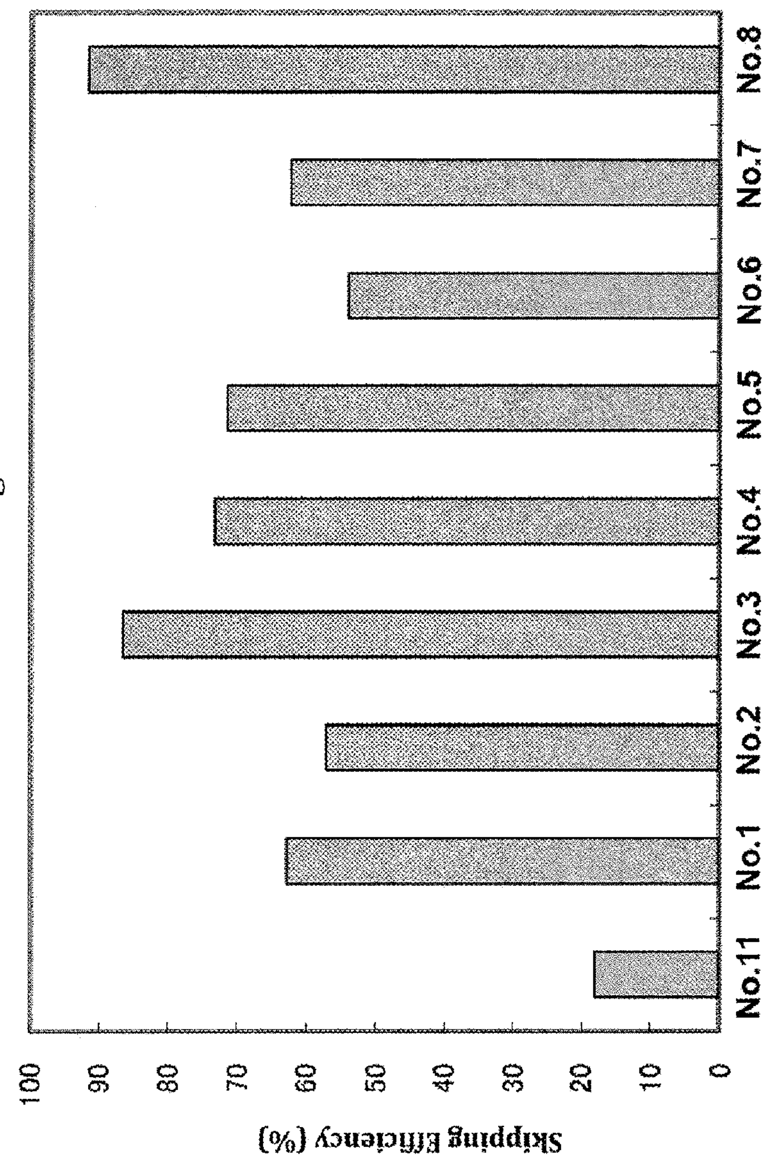
Figure 1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100
90
80
70
60
50
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No.4
No.5
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No.8

Figure 2

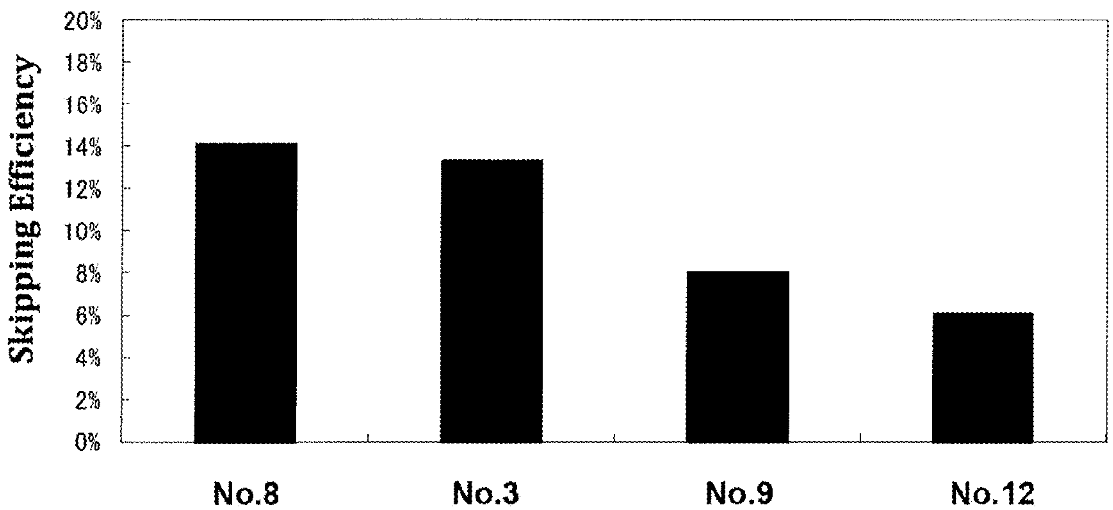
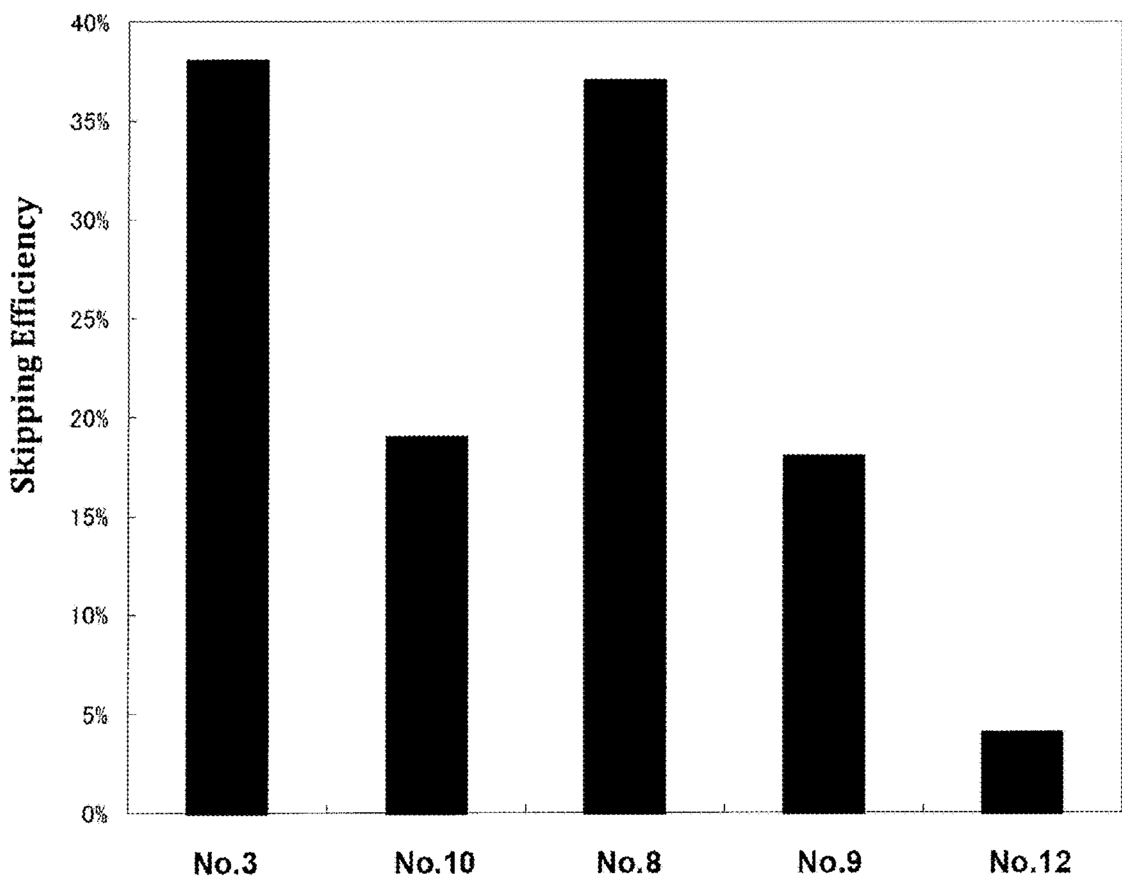


Figure 3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No.9
No.12

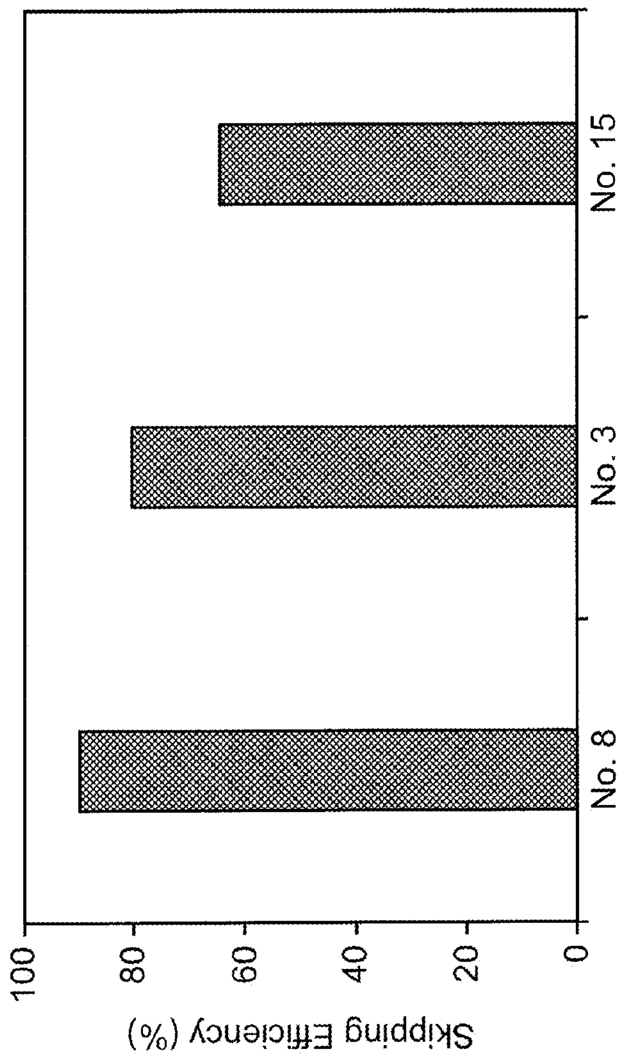


FIG. 4

Figure 5

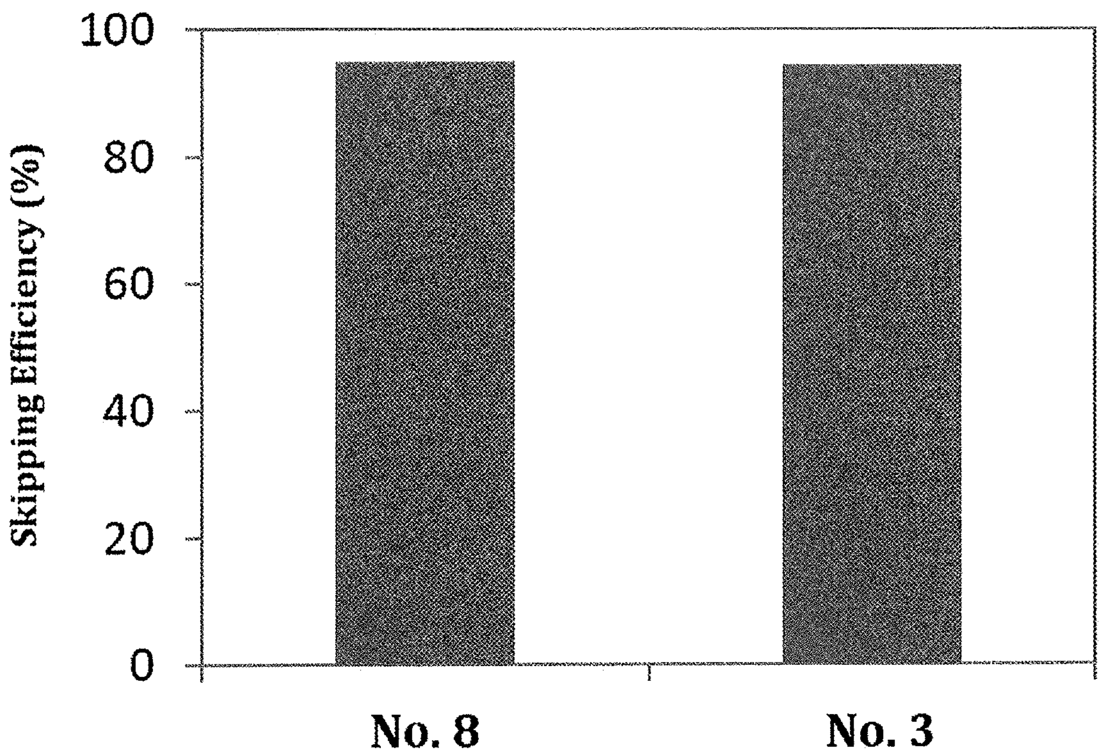
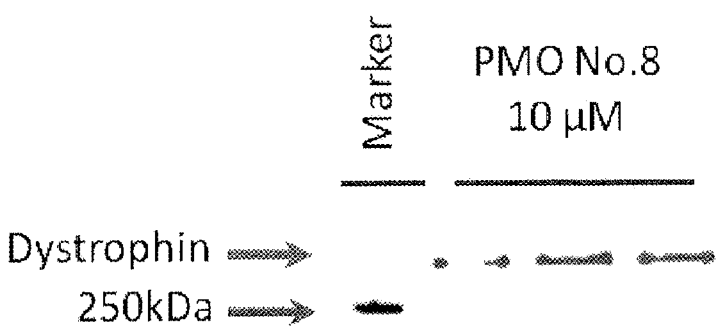
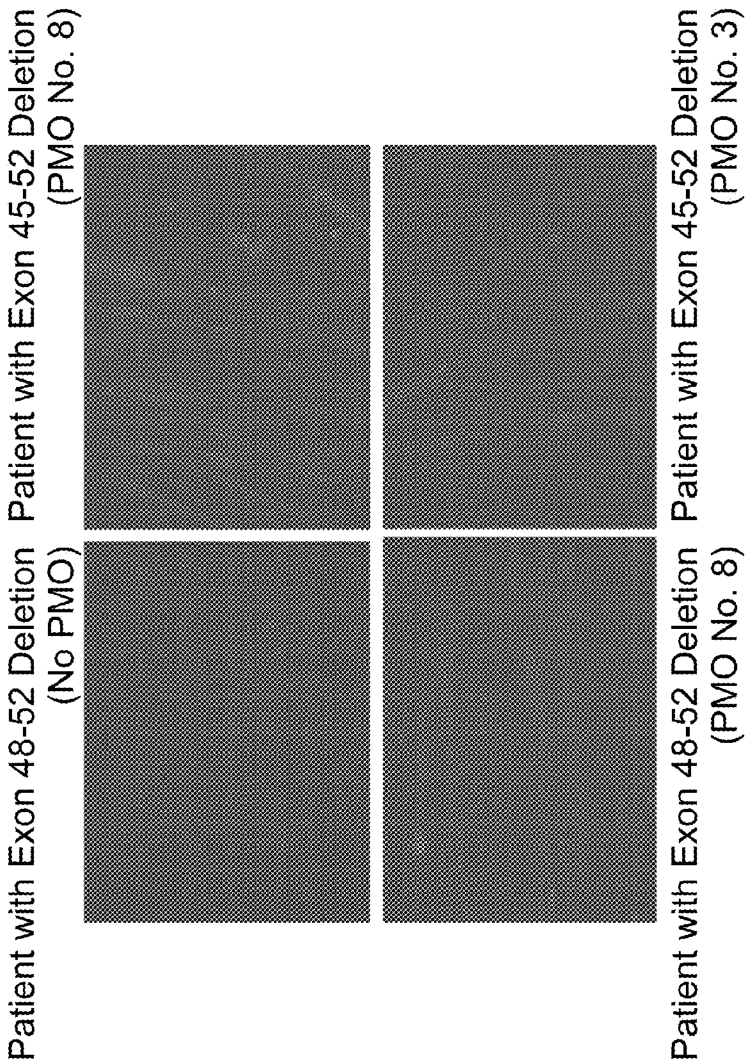


Figure 6

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(PMO No. 8)
Patient with Exon 48-52 Deletion
(No PMO)
Patient with Exon 45-52 Deletion
(PMO No. 3)
Patient with Exon 48-52 Deletion
(PMO No. 8)

FIG. 7

Figure 8

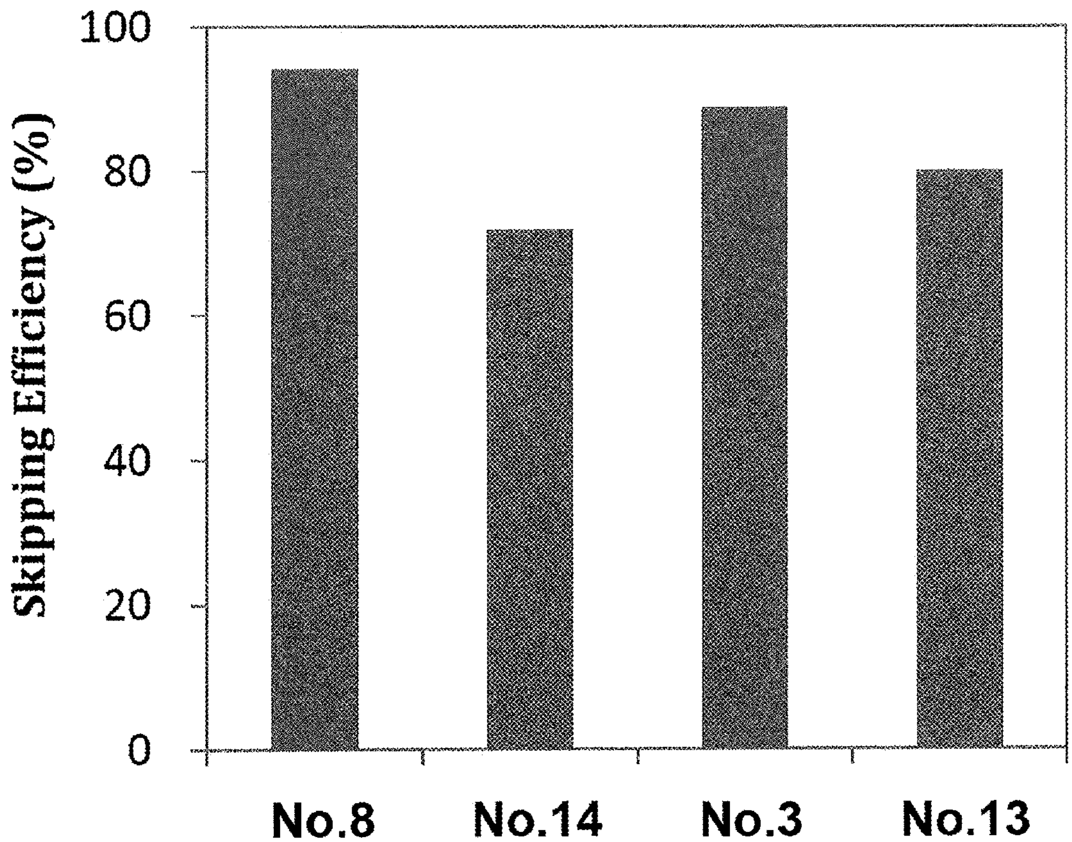


FIG. 9

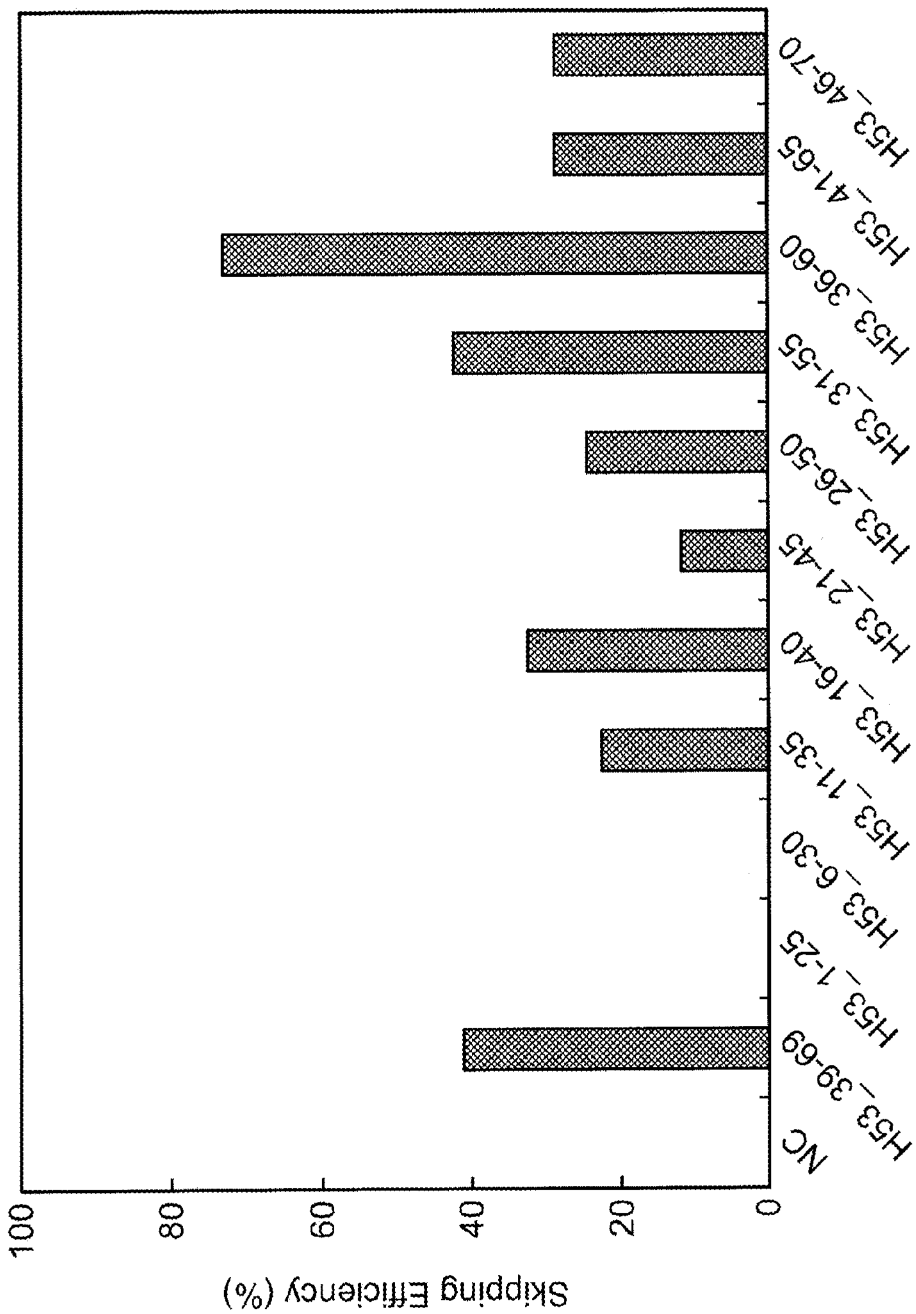

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_1-25
H53_6-30
H53_11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

FIG. 10

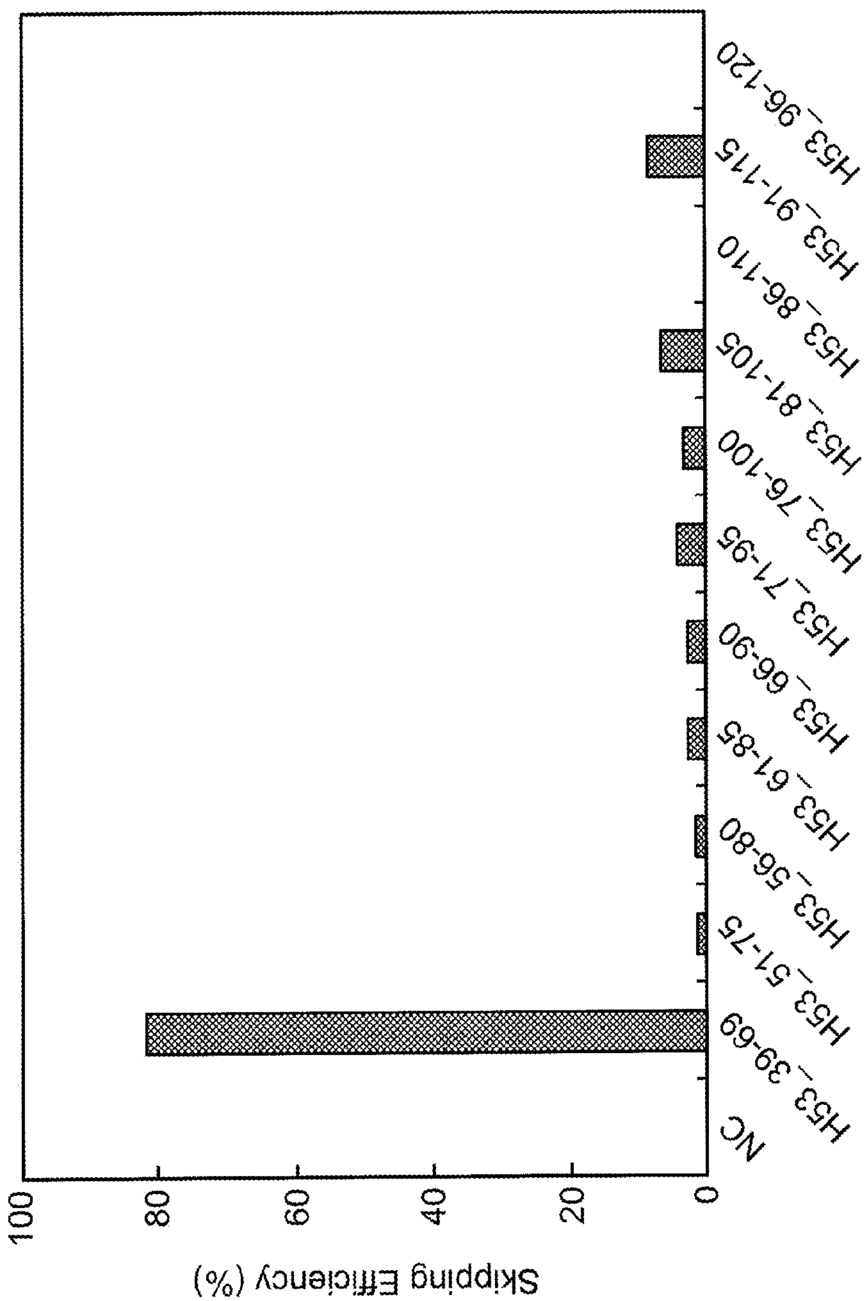

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_51-75
H53_56-80
H53_61-85
H53_66-90
H53_71-95
H53_76-100
H53_81-105
H53_86-110
H53_91-115
H53_96-120

FIG. 11

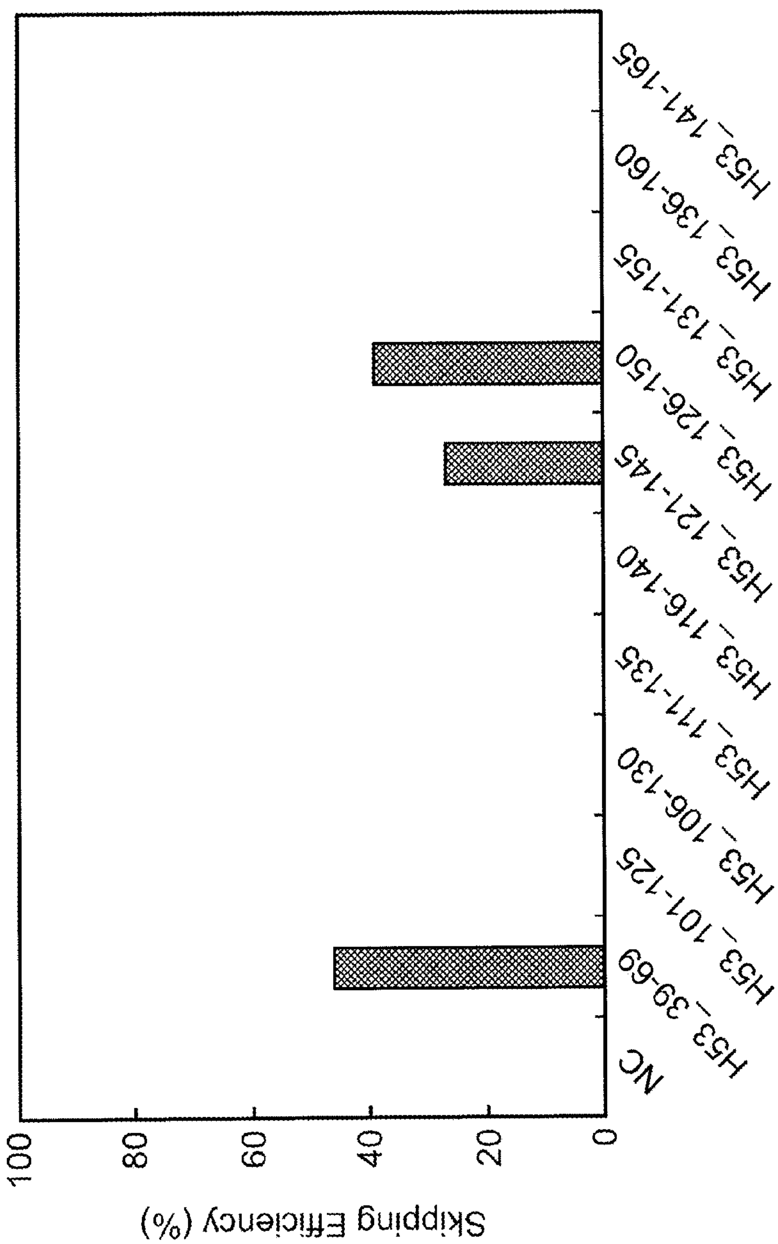

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_101-125
H53_106-130
H53_111-135
H53_116-140
H53_121-145
H53_126-150
H53_131-155
H53_136-160
H53_141-165

FIG. 12

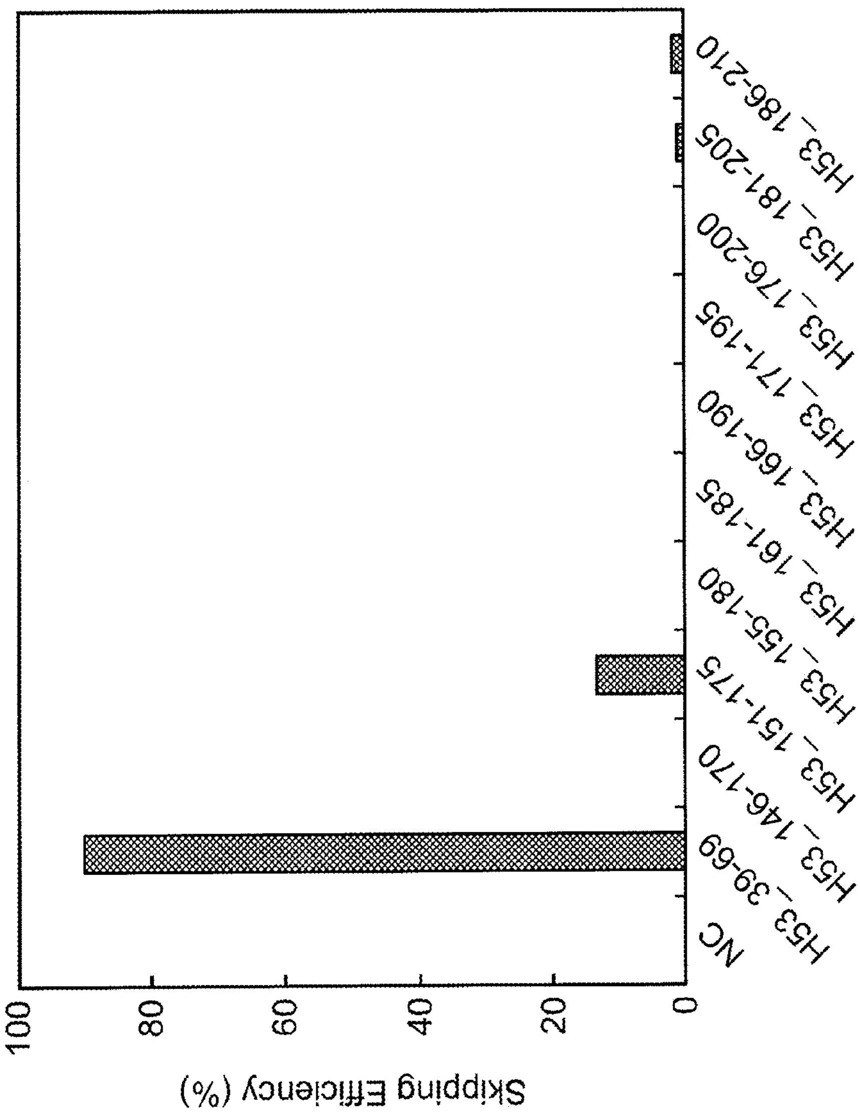

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_146-170
H53_151-175
H53_155-180
H53_161-185
H53_166-190
H53_171-195
H53_176-200
H53_181-205
H53_186-210

FIG. 13

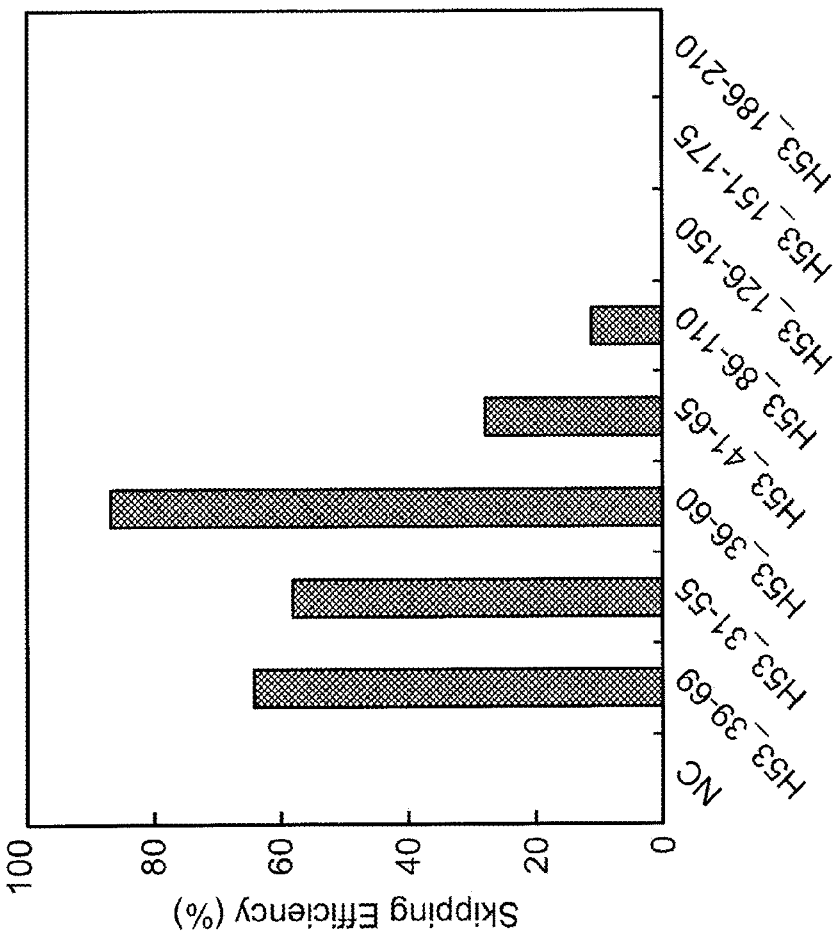
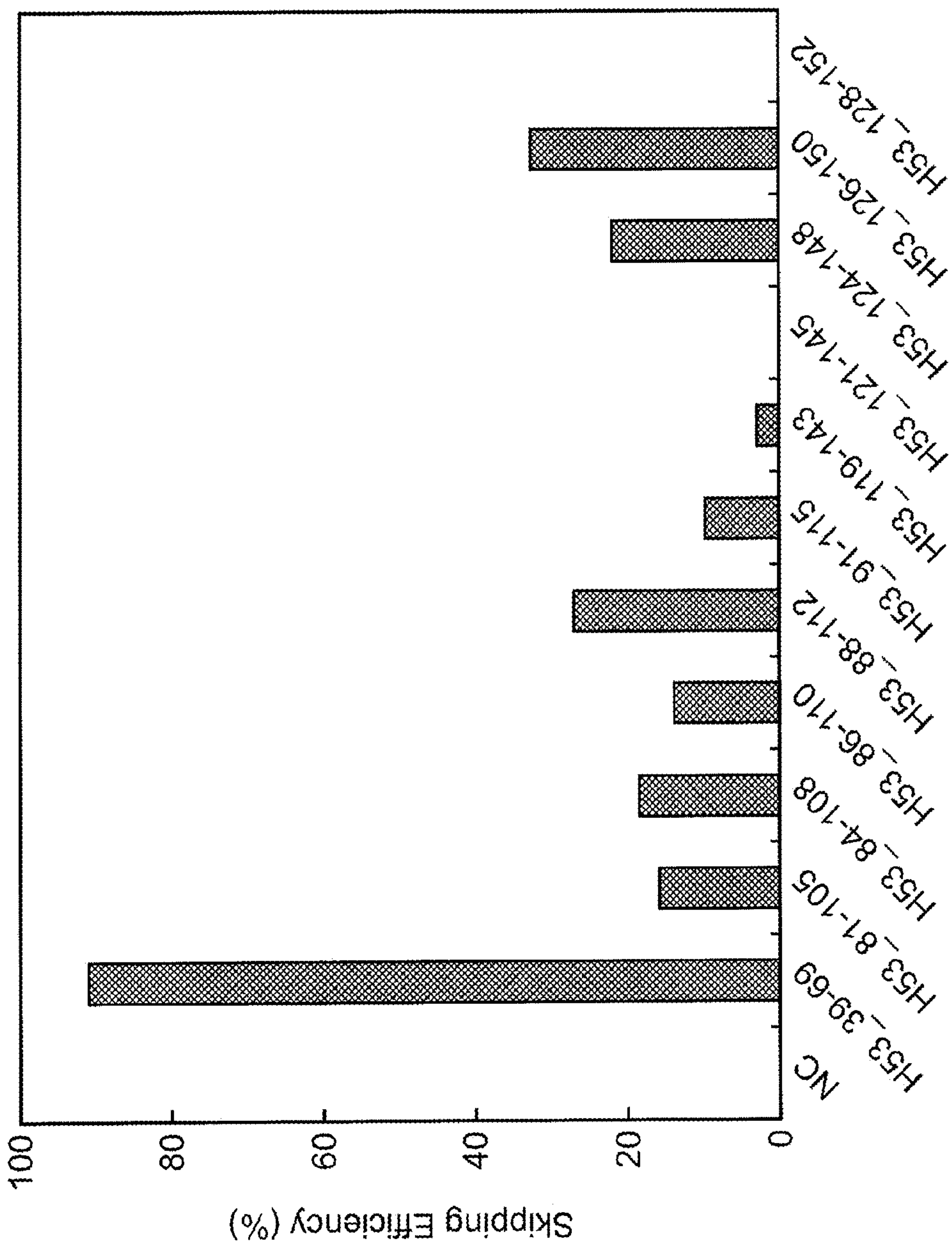

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_31-55
H53_36-60
H53_41-65
H53_86-110
H53_126-150
H53_151-175
H53_186-210

FIG. 14



Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_81-105
H53_84-108
H53_86-110
H53_88-112
H53_91-115
H53_119-143
H53_121-145
H53_124-148
H53_126-150
H53_128-152

FIG. 15

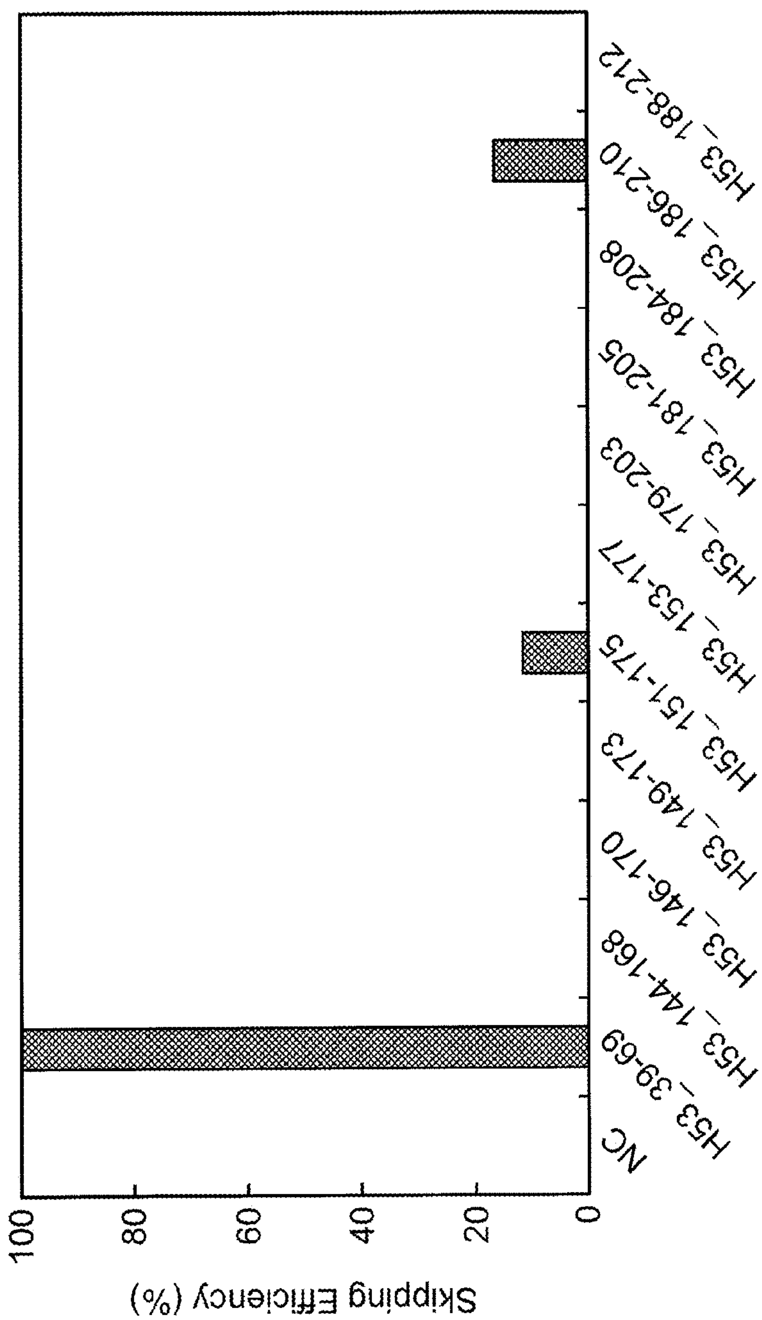

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_144-168
H53_146-170
H53_149-173
H53_151-175
H53_153-177
H53_179-203
H53_181-205
H53_184-208
H53_186-210
H53_188-212

FIG. 16

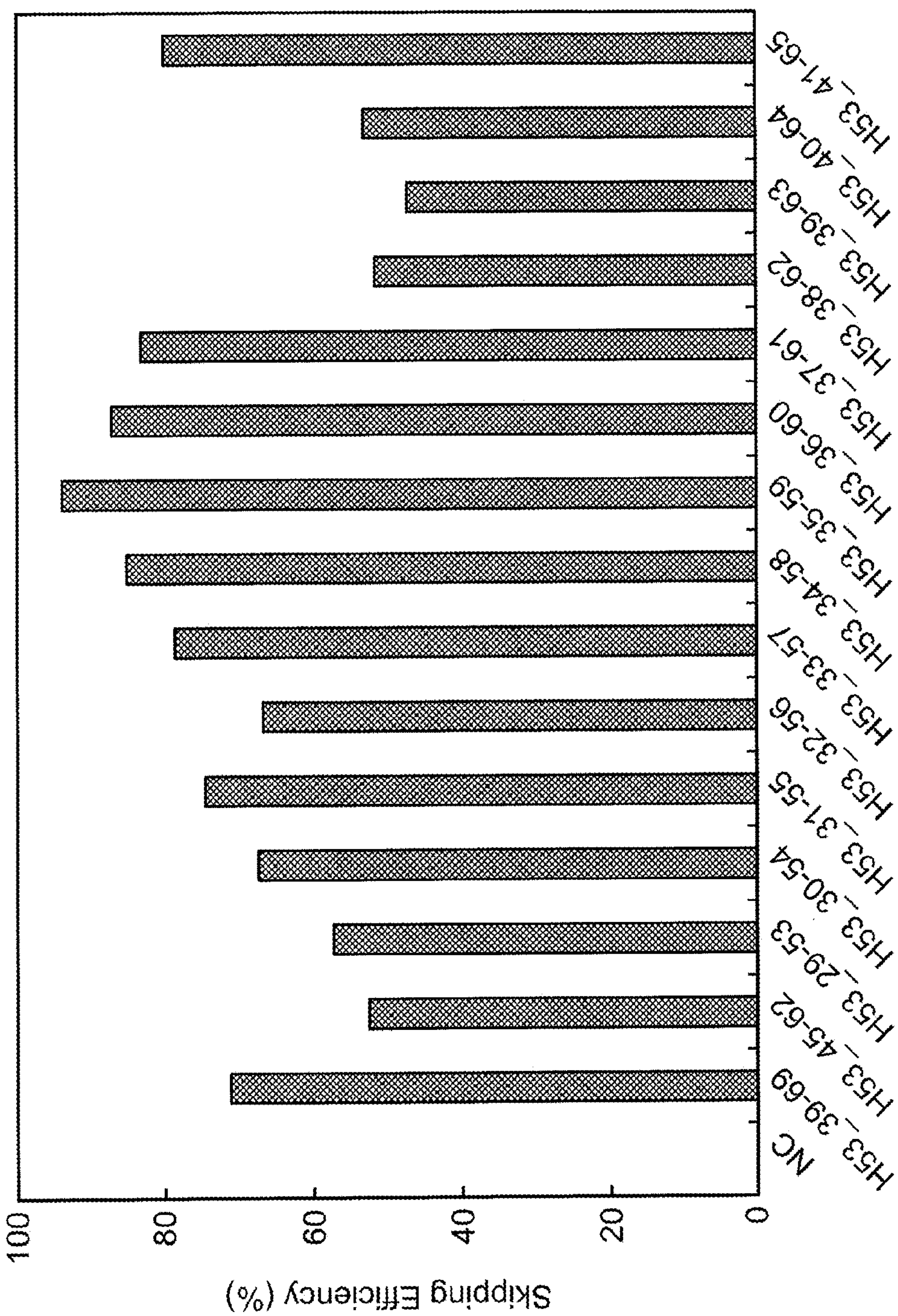
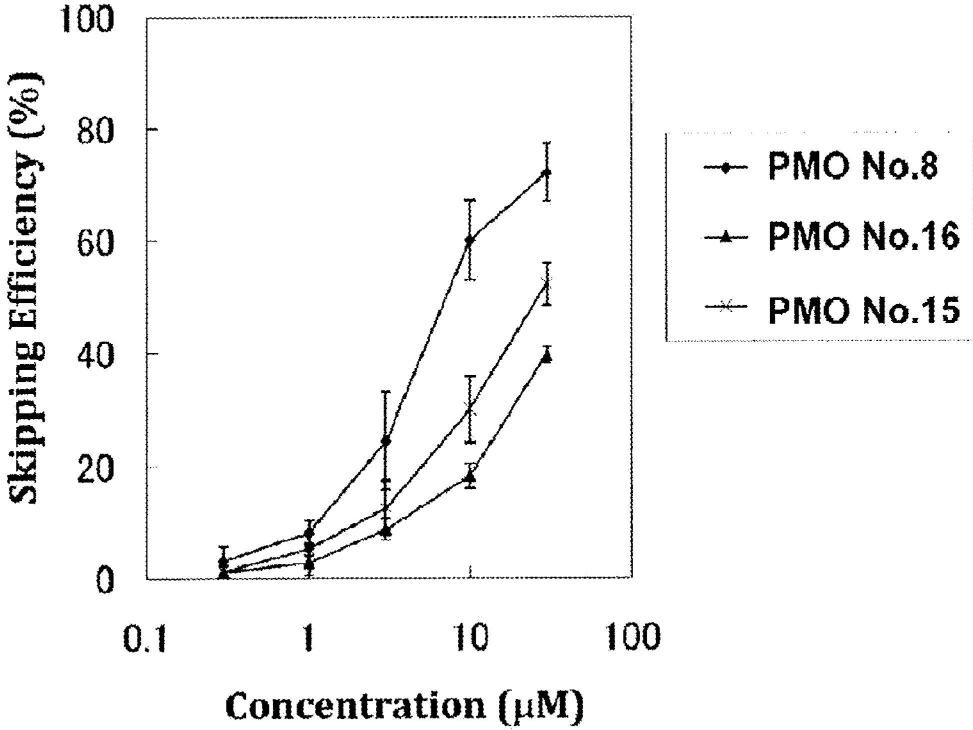

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_45-62
H53_29-53
H53_30-54
H53_31-55
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_38-62
H53_39-63
H53_40-64
H53_41-65

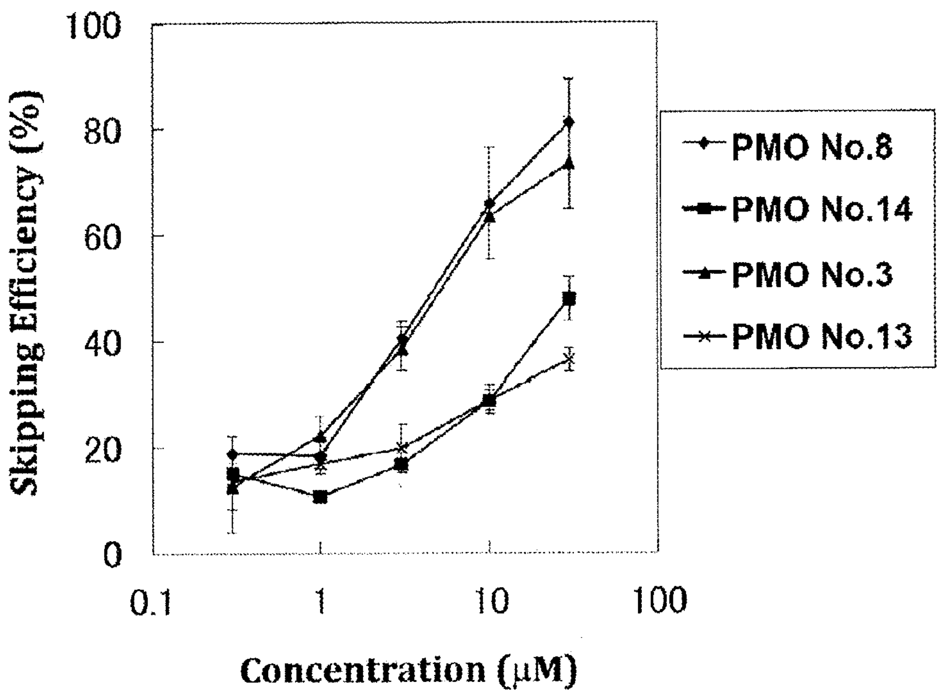
FIG. 17

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
H53_35-52
H53_38-55
H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59



100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 18



100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a Continuation of co-pending application Ser. No. 17/126,366, filed Dec. 18, 2020, which is a Continuation of application Ser. No. 16/408,529, filed May 10, 2019 (now U.S. Pat. No. 10,870,676 issued Dec. 22, 2020), which is a Continuation of application Ser. No. 15/619,996, filed Jun. 12, 2017 (now U.S. Pat. No. 10,329,319 issued Jun. 25, 2019), which is a Continuation of application Ser. No. 14/615,504, filed Feb. 6, 2015 (now U.S. Pat. No. 9,708,361 issued Jul. 18, 2017), which is a Continuation of application Ser. No. 13/819,520, filed Apr. 10, 2013 (now U.S. Pat. No. 9,079,934 issued Jul. 14, 2015), which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No. 2010-196032 filed Sep. 1, 2010, all of which are incorporated by reference in their entireties.

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Feb. 26, 2021, is named 209658_0001_11_subSL.txt and is 25,120 bytes in size.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are

typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., e t al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

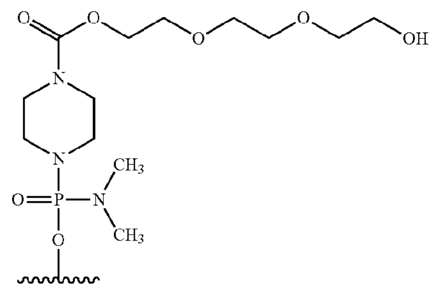
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

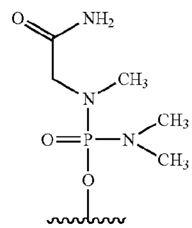
[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

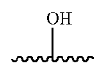
[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)



(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD

gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention

but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGT1TCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8

TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'-CCTCCGGTTCTGAAGGTG11TCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTICTIGT-3'	SEQ ID NO: 12
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTG'FTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGITCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTICTT-3'	SEQ ID NO: 20
34-54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTG1TC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oli-

gomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency }(\%)=A/(A+B)\times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl.

The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents. The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms.

Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

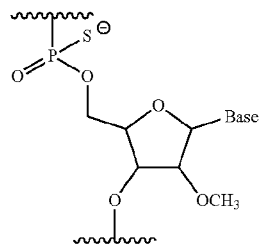
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

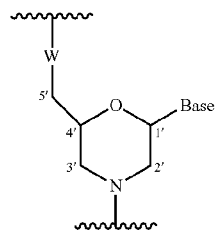
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

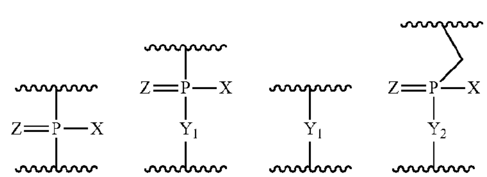
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein X represents —CH_2R^1, —O—CH_2R^1, —S—CH_2R^1, —NR_2R^3 or F;

R^1 represents H or an alkyl;

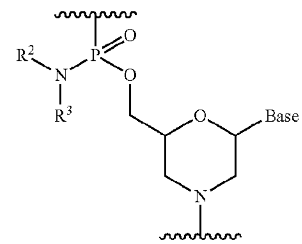
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).



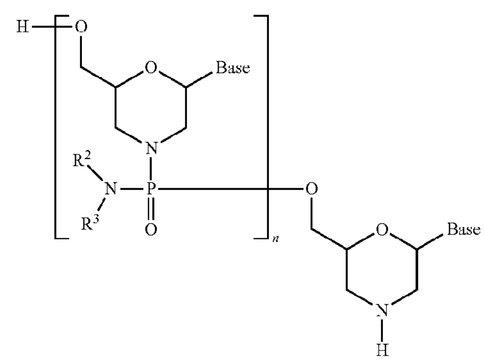
wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).

(I)



wherein Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

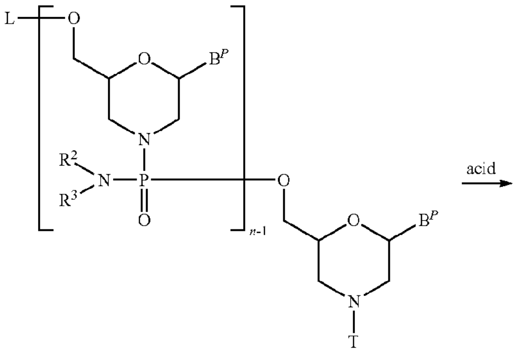
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

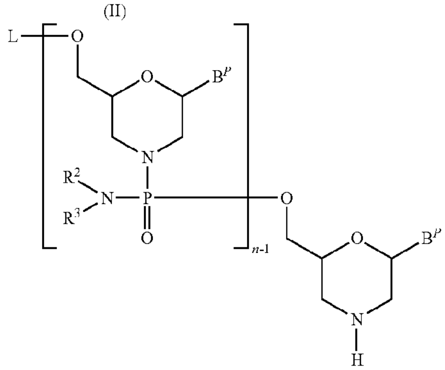
Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)



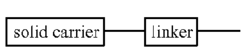
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin. HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2-PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (11) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

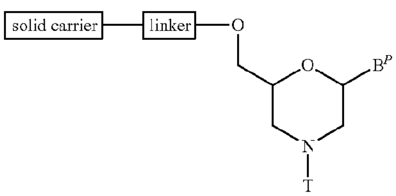
After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

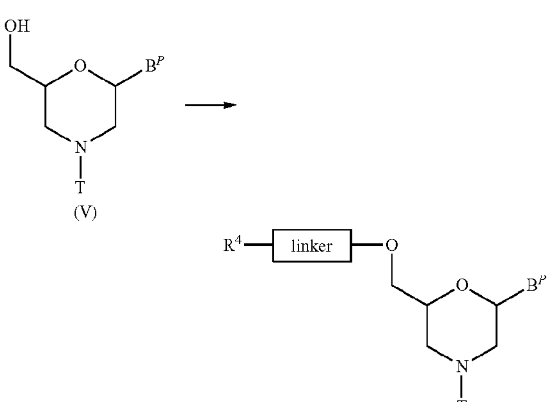
(IIa)



wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



(V)

(VI)

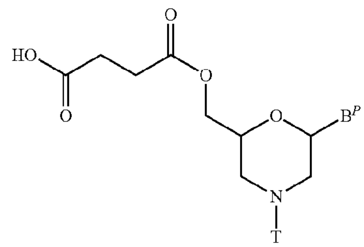
wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

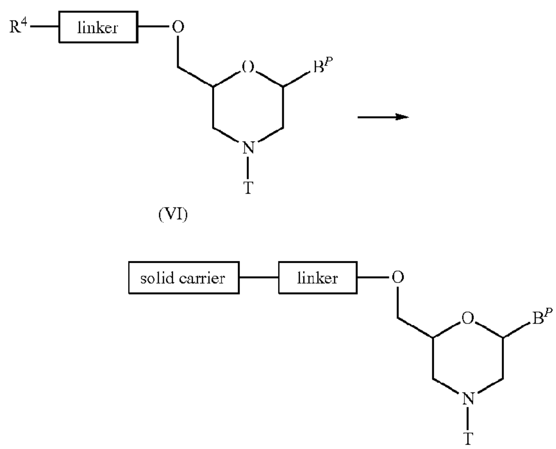
(VIa)



wherein B^P and T have the same significance as defined above.

Step 2:

Compound (VI) is reacted with a solid carcer by a condensing agent to prepare Compound (IIa).



(VI)

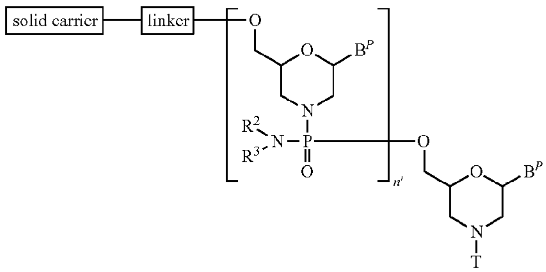
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)

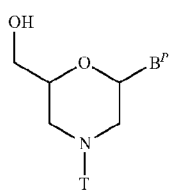


wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

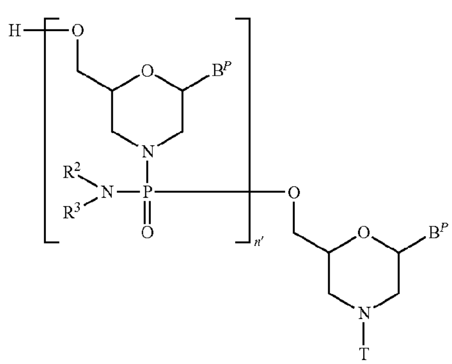
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

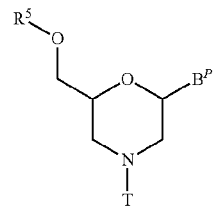
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

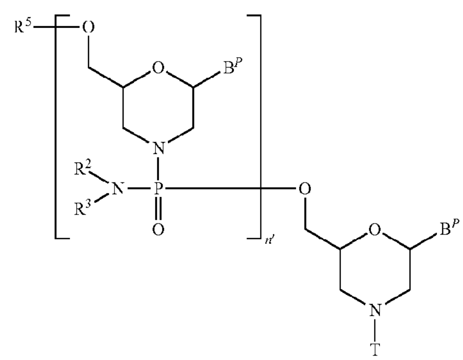


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

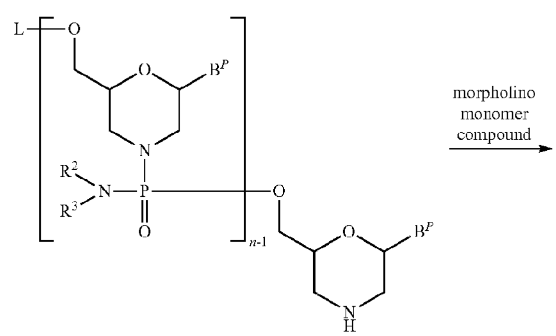
(IIc2)



wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

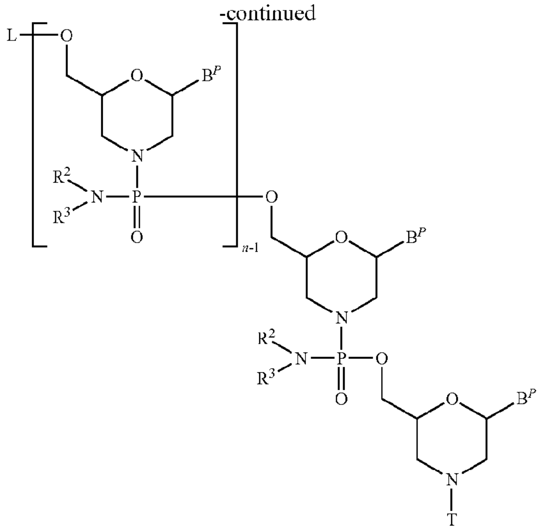
(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)

-continued



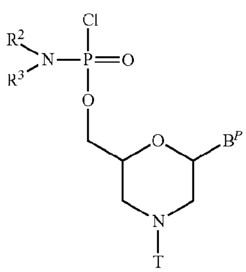
(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in

a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

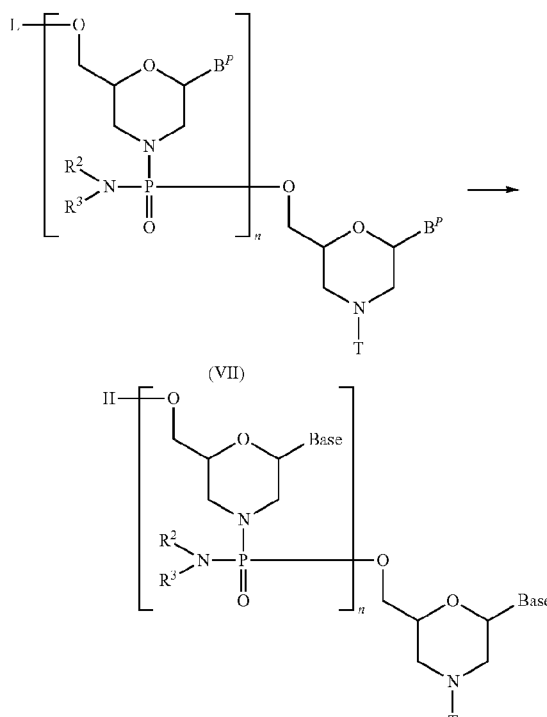
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)

(IX)

wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

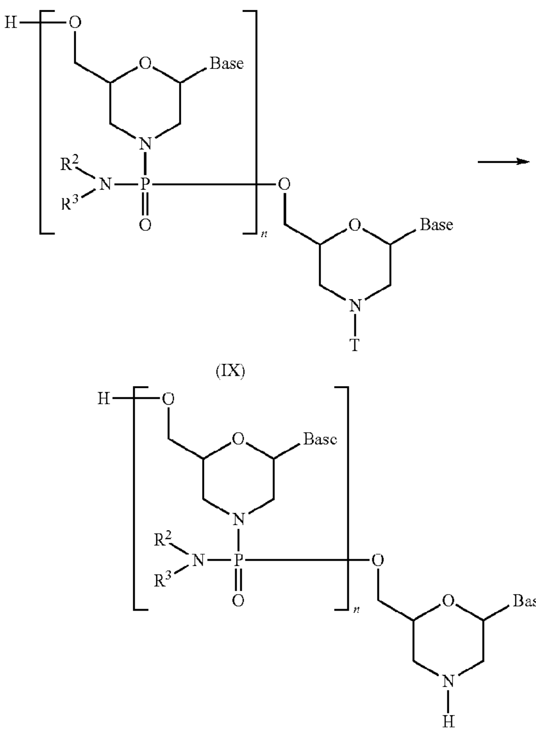
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)

(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used

is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

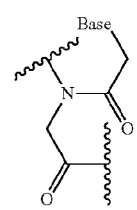
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

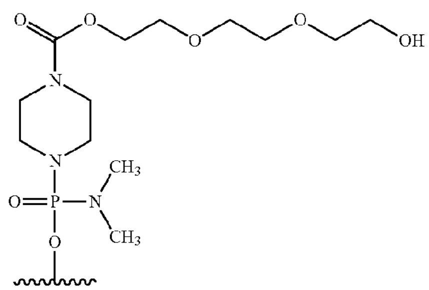
3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

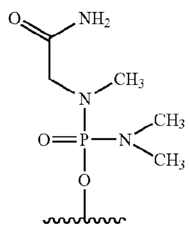
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.

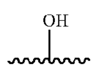
(1)



(2)



(3)



Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucos amine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethyl amine, dicyclohexylamine, N,N'-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl) aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and

p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable

additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine) One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

Reference Example 1

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]meth oxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-

(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV Measurement

Device: U-2910 (Hitachi, Ltd.)

Solvent: methanesulfonic acid

Wavelength: 265 nm

ε Value: 45000

Reference Example 2

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl] methoxy}butanoic Acid Loaded onto 2-aminomethylpolystyrene Resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 3

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4 (1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 4

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxy-

ethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, φ50× 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL

of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, φ40× 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82

Found: 6923.54

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96

Found: 8296.24

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13

Found: 7309.23

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94

Found: 8270.55

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13

Found: 7310.17

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94

Found: 8270.20

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01

Found: 5963.68

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55

Found: 6608.85

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11

Found: 7279.42

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95

Found: 8295.91

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15

Found: 7276.69

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27

Found: 8622.29

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63

Found: 10273.71

Comparative Example 2

PMO. No. 15 The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33

Found: 9940.77

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94

Found: 8238.69

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins; reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)

Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIG. **1**. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3' (SEQ ID NO: 45)

hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping Efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5×10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

hEx44F: 5'-TGTTGAGAAATGGCGGCGT-3' (SEQ ID NO: 48)

hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins

were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
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H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 mL of

Eagles minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 µM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 µl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 µl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)

Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

The reaction product, 1 µl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping Efficiency (\%)} = A/(A+B)\times100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed

at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MT Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

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<400> SEQUENCE: 39

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<210> SEQ ID NO 40
<211> LENGTH: 21
<212> TYPE: DNA
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<400> SEQUENCE: 40

aggatttgga acagaggcgt c 21

<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 41

gtctgccact ggcggaggtc 20

<210> SEQ ID NO 42
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<212> TYPE: DNA
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<400> SEQUENCE: 42

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catcaagcag aaggcaacaa 20

<210> SEQ ID NO 43
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 43

gaagtttcag ggccaagtca 20

<210> SEQ ID NO 44
<211> LENGTH: 963
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

atggagctac tgtcgccacc gctccgcgac gtagacctga cggcccccga cggctctctc 60

tgctcctttg ccacaacgga cgacttctat gacgacccgt gtttcgactc cccggacctg 120

cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaacccgaa 180

gagcactcgc acttccccgc ggcggtgcac ccggccccgg gcgcacgtga ggacgagcat 240

gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca 420

aaccagcggt tgcccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg 480

caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg 540

ccgggcccgc tgcccccggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagagcc ctgcggcgcc cgccctcctg ctggcggacg tgccttctga gtcgcctccg 840

cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca 900

ccggacgccg ccccgcagtg ccctgcgggt gcgaaccccca acccgatata ccaggtgctc 960

tga 963

<210> SEQ ID NO 45
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<400> SEQUENCE: 45

cgggcttgga cagaacttac 20

<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

<210> SEQ ID NO 47
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgaaggtgt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 50

ucccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
<211> LENGTH: 25
<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 52

uuguacuuca ucccacugau ucuga 25

<210> SEQ ID NO 53
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<212> TYPE: RNA
<213> ORGANISM: Artificial

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 54

gaagguguuc uuguacuuca uccca 25

<210> SEQ ID NO 55
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 55

guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 59

ucauucaacu guugccuccg guucu 25

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 60

acauuucauu caacuguugc cuccg                                            25


<210> SEQ ID NO 61
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc                                            25


<210> SEQ ID NO 62
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 62

gaauccuuua acauuucauu caacu                                            25


<210> SEQ ID NO 63
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu                                            25


<210> SEQ ID NO 64
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 64

ccauuguguu gaauccuuua acauu                                            25


<210> SEQ ID NO 65
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua                                            25


<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid
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<400> SEQUENCE: 66

uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 67

uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 68

gcuucuuccu uagcuuccag ccauu 25

<210> SEQ ID NO 69
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 69

gcucagcuuc uuccuuagcu uccag 25

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73

<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

uuggcucugg ccuguccuaa gaccu 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa 25

<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 77

uuccaugacu caagcuuggc ucugg 25

<210> SEQ ID NO 78
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc 25

<210> SEQ ID NO 79
<211> LENGTH: 25
<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc 25

<210> SEQ ID NO 80
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 80

guauagggac ccuccuucca ugacu 25

<210> SEQ ID NO 81
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 81

cuacuguaua gggacccucc uucca 25

<210> SEQ ID NO 82
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuuuuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 85

gauuuucuuu uggauugcau cuacu 25

<210> SEQ ID NO 86
<211> LENGTH: 25
<212> TYPE: RNA

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 86

ucugugauuu ucuuuuggau ugcau 25

<210> SEQ ID NO 87
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 87

ugguuucugu gauuuucuuu uggau 25

<210> SEQ ID NO 88
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 88

ccuuagcuuc cagccauugu guuga 25

<210> SEQ ID NO 89
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 89

ucuuccuuag cuuccagcca uugug 25

<210> SEQ ID NO 90
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 90

ggcucuggcc uguccuaaga ccugc 25

<210> SEQ ID NO 91
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 91

agcuuggcuc uggccugucc uaaga 25

<210> SEQ ID NO 92
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 92

cucaagcuug gcucuggccu guccu 25

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 93

gacccuccuu ccaugacuca agcuu 25

<210> SEQ ID NO 94
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 94

auagggaccc uccuuccaug acuca 25

<210> SEQ ID NO 95
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 95

cuguauaggg acccuccuuc cauga 25

<210> SEQ ID NO 96
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 96

ugugauuuuc uuuuggauug caucu 25

<210> SEQ ID NO 97
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 97

guuucuguga uuuucuuuug gauug 25

<210> SEQ ID NO 98
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 98

cuugguuucu gugauuuucu uuugg 25

<210> SEQ ID NO 99
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 99

ccgguucuga agguguucuu guacu 25

<210> SEQ ID NO 100
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 100

uccgguucug aagguguucu uguac 25

<210> SEQ ID NO 101
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 101

ccuccgguuc ugaagguguu cuugu 25

<210> SEQ ID NO 102
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 102

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<210> SEQ ID NO 103
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 103

ugccuccggu ucugaaggug uucuu 25

<210> SEQ ID NO 104
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 104

uugccuccgg uucugaaggu guucu 25

<210> SEQ ID NO 105
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 105

uguugccucc gguucugaag guguu 25

<210> SEQ ID NO 106
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 106

cuguugccuc cgguucugaa ggugu 25

<210> SEQ ID NO 107
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 107

acuguugccu ccgguucuga aggug 25

<210> SEQ ID NO 108
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 108

aacuguugcc uccgguucug aaggu 25

<210> SEQ ID NO 109
<211> LENGTH: 30
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 109

uguugccucc gguucugaag guguucuugu 30

<210> SEQ ID NO 110
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 110

gguucugaag guguucuugu 20

<210> SEQ ID NO 111
<211> LENGTH: 20
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 111

uccgguucug aagguguucu 20

<210> SEQ ID NO 112
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 112

ccuccgguuc ugaagguguu 20

<210> SEQ ID NO 113
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 113

uugccuccgg uucugaaggu 20

<210> SEQ ID NO 114
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 114

uguugccucc gguucugaag 20

<210> SEQ ID NO 115
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 115

uucugaaggu guucuugu 18

<210> SEQ ID NO 116
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 116

cgguucugaa gguguucu 18

<210> SEQ ID NO 117
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 117

cuccgguucu gaaggugu 18

<210> SEQ ID NO 118
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 118

ugccuccggu ucugaagg 18

<210> SEQ ID NO 119
<211> LENGTH: 18

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 119

uguugccucc gguucuga                                          18


<210> SEQ ID NO 120
<211> LENGTH: 15
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 120

uucugaaggu guucu                                             15


<210> SEQ ID NO 121
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 121

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<210> SEQ ID NO 122
<211> LENGTH: 15
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 122

uugccuccgg uucug                                             15


<210> SEQ ID NO 123
<211> LENGTH: 18
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 123

cuguugccuc cgguucug                                          18


<210> SEQ ID NO 124
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(25)
<223> OTHER INFORMATION: nucleotides 36 to 60 of exon 53 of wild-type
      dystrophin gene

<400> SEQUENCE: 124

gaacaccuuc agaaccggag gcaac                                  25
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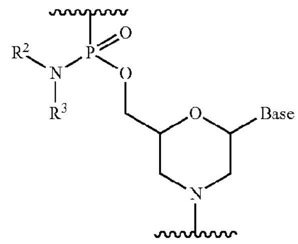
The invention claimed is:

1. A composition comprising a cationic polymer with a phosphorodiamidate morpholino oligomer (PMO) antisense oligomer that is 100% complementary to a target sequence of 5'-GAACACCUUCAGAACCGGAGGCAAC-3' (SEQ ID NO: 124),

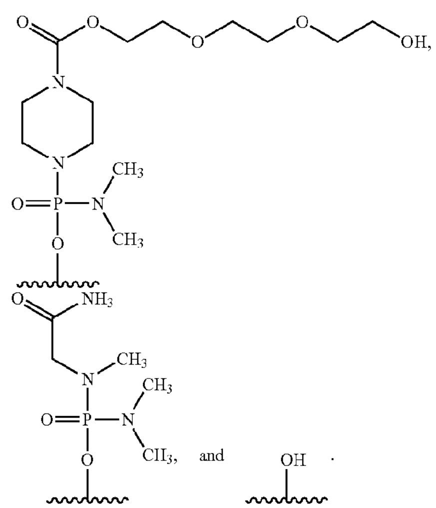
wherein said PMO antisense oligomer causes skipping of the 53rd exon in a human dystrophin pre-mRNA,

wherein said PMO antisense oligomer hybridizes to said target sequence with Watson-Crick base pairing under physiological conditions,

wherein said PMO antisense oligomer has exactly 25 phosphorodiamidate morpholino monomers, each of which has the formula:



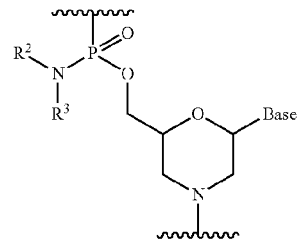
wherein each of R^2 and R^3 represents a methyl, wherein Base is a nucleobase selected from the group consisting of uracil, cytosine, thymine, adenine, and guanine, and wherein the 5' end of said PMO antisense oligomer has a formula selected from the group consisting of:



2. A composition that causes skipping of the 53rd exon in a human dystrophin pre-mRNA, comprising a cationic carrier with a 25-mer phosphorodiamidate morpholino oligomer (PMO) antisense oligomer,

wherein said PMO antisense oligomer is 100% complementary to a target sequence of 5'-GAACACCUUCAGAACCGGAGGCAAC-3' (SEQ ID NO: 124),

wherein each phosphorodiamidate morpholino monomer of said PMO antisense oligomer has the formula:



wherein each of R^2 and R^3 represents a methyl, wherein Base is a nucleobase selected from the group consisting of uracil, cytosine, thymine, adenine, and guanine, and

wherein said cationic carrier promotes delivery of said composition to a muscle tissue.

3. The composition of claim **2**, wherein said cationic carrier is a cationic polymer.

4. A composition comprising:

a) a 25-mer phosphorodiamidate morpholino oligomer (PMO) antisense oligomer that is 100% complementary to a target sequence of 5'-GAACACCUUCAGAACCGGAGGCAAC-3' (SEQ ID NO: 124), wherein said PMO antisense oligomer causes skipping of the 53rd exon in a human dystrophin pre-mRNA; and

b) a carrier to promote delivery of said PMO antisense oligomer to a muscle tissue.

5. The composition of claim **4**, wherein said carrier is a cationic polymer.

* * * * *

EXHIBIT AM

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
17/126,366	12/18/2020		2840	209658-0001-10-US-604420	13	1

CONFIRMATION NO. 7560

FILING RECEIPT

OC000000124025242

55694
FAEGRE DRINKER BIDDLE & REATH LLP (DC)
1500 K STREET, N.W.
SUITE 1100
WASHINGTON, DC 20005-1209

Date Mailed: 03/12/2021

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Inventor(s)

Naoki WATANABE, Tsukuba-shi, JAPAN;
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Assignment For Published Patent Application

NIPPON SHINYAKU CO., LTD., Kyoto-shi, JAPAN
NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY, Kodaira-shi, JAPAN

Power of Attorney: The patent practitioners associated with Customer Number 055694

Domestic Priority data as claimed by applicant

This application is a CON of 16/408,529 05/10/2019 PAT 10870676
which is a CON of 15/619,996 06/12/2017 PAT 10329319
which is a CON of 14/615,504 02/06/2015 PAT 9708361
which is a CON of 13/819,520 04/10/2013 PAT 9079934
which is a 371 of PCT/JP2011/070318 08/31/2011

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JAPAN 2010-196032 09/01/2010 No Access Code Provided

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/126,366**

Projected Publication Date: 06/17/2021

Non-Publication Request: No

Early Publication Request: No
Title

ANTISENSE NUCLEIC ACIDS

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Substitute for form 1449/PTO				***Complete if Known***	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	17/126,366
				Filing Date	August 31, 2011
				First Named Inventor	Naoki WATANABE
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	Not Yet Assigned
Sheet	1	of	6	Attorney Docket Number	209658-0001-10-US-604420

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A	US-2003/0166588-A1	09-04-2003	Iversen et al.	
	B	US-2007/0082861-A1	04-12-2007	Matsuo et al.	
	C	US-2009/0131624-A1	05-21-2009	REEVES et al.	
	D	US-2009/0088562-A1	04-02-2009	Weller et al.	
	E	US-5,185,444	02-09-1993	Summerton et al.	
	F	US-5,217,866	06-08-1993	Summerton et al.	
	G	US-2009/0131632-A1	05-21-2009	Fox et al.	
	H	US-5,142,047	08-25-1992	Summerton et al.	
	I	US-7,935,816	05-03-2011	Li	

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Sheet 2 of 6

NON PATENT LITERATURE DOCUMENTS

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	A	Sazani et al., "Safety Pharmacology and Genotoxicity Evaluation of AVI-4658," Int. J. Toxicol. (2010) 29(2):143-156	
	B	Bushby et al., "Diagnosis and Management of Duchenne Muscular Dystrophy, Part 1: Diagnosis, and Pharmacological and Psychosocial Management," Lancet Neurol. (2010) 9(1): 77-93	
	C	Kinali et al., "Local Restoration of Dystrophin Expression with the Morpholino Oligomer AVI-4658 in Duchenne Muscular Dystrophy: A Single-Blind, Placebo-Controlled, Dose-Escalation, Proof-of-Concept Study," Lancet Neurol. (2009) 8(10): 918-928	
	D	Hoffman et al., "Skipping Toward Personalized Molecular Medicine," N. Engl. J. Med. (2007) 357(26): 2719-2722	
	E	Wilton et al., "Exon Skipping and Duchenne Muscular Dystrophy: Hope, Hype and How Feasible?" Neurol. India (2008) 56(3): 254-262	
	F	Ginjaar et al., "Dystrophin Nonsense Mutation Induces Different Levels of Exon 29 Skipping and Leads to Variable Phenotypes Within One BMD Family," Eur. J. Hum. Genet. (2000) 8(10): 793-796	
	G	Muntoni et al., "The Development of Antisense Oligonucleotide Therapies for Duchenne Muscular Dystrophy: Report on a TREAT-NMD Workshop Hosted by the European Medicines Agency (EMA), on September 25th 2009," Neuromuscul. Disord. (2010) 20(5):	
	H	Goemans et al., "Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy," N. Engl. J. Med. (2011) 364(16): 1513-1522	
	I	Cirak et al., "Exon Skipping and Dystrophin Restoration in Patients with Duchenne Muscular Dystrophy After Systemic Phosphorodiamidate Morpholino Oligomer Treatment: An Open-Label, Phase 2, Dose-Escalation Study," Lancet (2011) 378(9791): 595-605	
	J	Nakamura et al., "Exon-Skipping Therapy for Duchenne Muscular Dystrophy," Neuropathology (2009) 29(4): 494-501	

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	K	Yokota et al., "A Renaissance for Antisense Oligonucleotide Drugs in Neurology," Arch. Neurol. (2009) 66(1): 32-38	
	L	Aartsma-Rus et al., "Less is More: Therapeutic Exon Skipping for Duchenne Muscular Dystrophy," Lancet Neurol. (2009) 8(10): 873-875	
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	N	Moulton et al., "Gene Knockdowns in Adult Animals: PPMOs and Vivo-Morpholinos," Molecules (2009) 14(3): 1304-1323	
	O	Summerton, "Morpholino Antisense Oligomers: The Case for an RNase H-independent Structural Type," Biochim. Biophys. Acta (1999) 1489(1): 141-158	
	P	Muntoni et al., "149th ENMC International Workshop and 1st TREAT-NMD Workshop on: "Planning Phase I/II Clinical Trials Using Systemically Delivered Antisense Oligonucleotides in Duchenne Muscular Dystrophy," Neuromuscul. Disord. (2008) 18(3): 268-275	
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	S	Final Office Action dated Nov 16, 2017 in USSN 14/776,533	
	T	Express Abandonment filed March 20, 2019 and Notice of Abandonment dated April 5, 2019 in USSN 14/776,533	

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				Filing Date	August 31, 2011
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				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	Not Yet Assigned
Sheet	4	of	6	Attorney Docket Number	209658-0001-10-US-604420

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Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	U	McClorey et al., "Induced Dystrophin Exon Skipping in Human Muscle Explants," Neuromuscul. Disord. (2006) 16(9-10): 583-590	
	V	U.S. Provisional Application No. 61/591,354	
	W	Alberts et al., Molecular Biology of the Cell 191-234, 299-374 (4th ed. 2002)	
	X	Wood and Douglas, "Splicing therapy for neuromuscular disease," Mol. and Cellular Neuroscience (2013) 56: 169-185	
	Y	Dominski and Kole, "Restoration of Correct Splicing in Thalassemic pre-mRNA by Antisense Oligonucleotides," Proc. Natl. Acad. Sci. U.S.A. (1993) 90: 8673-8677	
	Z	Dominski and Kole, "Identification and Characterization by Antisense Oligonucleotides of Exon and Intron Sequences Required for Splicing," Mol. Cell. Biol. (1994) 14(11): 7445-7454	
	AA	Sierakowska et al., "Repair of Thalassemic Human beta-Globin mRNA in Mammalian Cells by Antisense Oligonucleotides," Proc. Natl. Acad. Sci. U.S.A. (1996) 93: 12840-12844	
	AB	MacCoss et al., "Facile detritylation of nucleoside derivatives by using trifluoracetic acid," Carbohydrate Res 60(1): 206-209 (1978)	
	AC	Experimental Report submitted March 16, 2018 in EP 3018211	
	AD	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01135 (USPN 10,385,092)	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 5 of 6

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	AE	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01134 (USPN 9,708,361)	
	AF	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01136 (USPN 10,407,461)	
	AG	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01137 (USPN 10,487,106)	
	AH	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01138 (USPN 10,647,741)	
	AI	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01139 (USPN 10,662,217)	
	AJ	Declaration of Kelley M. Hayes Greenhill, submitted June 21, 2021 in IPR2021-01140 (USPN 10,683,322)	
	AK	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01135 (USPN 10,385,092)	
	AL	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01134 (USPN 9,708,361)	
	AM	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01136 (USPN 10,407,461)	
	AN	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01137 (USPN 10,487,106)	

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				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	Not Yet Assigned
Sheet	6	of	6	Attorney Docket Number	209658-0001-10-US-604420

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	AP	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01139 (USPN 10,662,217)	
	AQ	Declaration of Dr. David R. Corey, submitted June 21, 2021 in IPR2021-01140 (USPN 10,683,322)	
	AR	Petition for Inter Partes Review, Case No. IPR2021-01134 (USPN 9,708,361), filed June 21, 2021	
	AS	Petition for Inter Partes Review, Case No. IPR2021-01135 (USPN 10,385,092), filed June 21, 2021	
	AT	Petition for Inter Partes Review, Case No. IPR2021-01136 (USPN 10,407,461), filed June 21, 2021	
	AU	Petition for Inter Partes Review, Case No. IPR2021-01137 (USPN 10,487,106), filed June 21, 2021	
	AV	Petition for Inter Partes Review, Case No. IPR2021-01138 (USPN 10,647,741), filed June 21, 2021	
	AW	Petition for Inter Partes Review, Case No. IPR2021-01139 (USPN 10,662,217), filed June 21, 2021	
	AX	Petition for Inter Partes Review, Case No. IPR2021-01140 (USPN 10,683,322), filed June 21, 2021	

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ATTORNEY DOCKET NO. 209658-0001-10-US-604420
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 17/126,366 Confirmation No.: 7560

Filed: August 31, 2011 Art Unit: 1635

For: ANTISENSE NUCLEIC ACIDS Examiner: Not Yet Assigned

INFORMATION DISCLOSURE STATEMENT (IDS)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

☒ **Under 37 C.F.R. § 1.97(b):** Pursuant to 37 C.F.R. §§ 1.56 and 1.97(b), Applicant brings to the attention of the Examiner the documents listed on the attached PTO/SB/08 Form. To the undersigned's knowledge, this IDS is being filed before the mailing date of a first Office Action on the merits, before the mailing date of a first Office Action on the merits after filing an RCE under § 1.114, or within three months of the application filing date.

☐ **Under 37 C.F.R. § 1.97(c):** Pursuant to 37 C.F.R. §§ 1.56 and 1.97(c), Applicant brings to the attention of the Examiner the documents listed on the attached PTO/SB/08 Form. This IDS is being filed after the events recited in § 1.97(b) but, to the undersigned's knowledge, before the mailing date of a Final Office Action, a Notice of Allowance, or another action that closes prosecution in the application.

☐ The fee of $260.00 set forth in § 1.17(p) is included herein;

☐ Applicant submits that each item of information contained in this IDS was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this IDS. 37 C.F.R. § 1.97(e)(1).

☐ Applicant submits that no item of information contained in this IDS was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in this IDS was known to any individual designated in § 1.56(c) more than three months prior to the filing of this IDS.
37 C.F.R. § 1.97(e)(2).

☐ **<u>Under 37 C.F.R. § 1.97(d)</u>:** Pursuant to 37 C.F.R. §§ 1.56 and 1.97(d), Applicant brings to the attention of the Examiner the documents listed on the attached PTO/SB/08 Form. This IDS is being filed after the events recited in § 1.97(c) but before payment of the issue fee.

☐ The fee of $260.00 set forth in § 1.17(p) is included herein; and

☐ Applicant submits that each item of information contained in this IDS was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this IDS.

☐ Applicant submits that no item of information contained in this IDS was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in this IDS was known to any individual designated in § 1.56(c) more than three months prior to the filing of this IDS.
37 C.F.R. § 1.97(e)(2).

☐ **<u>Under 37 C.F.R. § 1.97(i)</u>:** Pursuant to 37 C.F.R. §§ 1.56 and 1.97(i), Applicant brings to the attention of the Examiner the documents listed on the attached PTO/SB/08 Form. This IDS is being filed after the events recited in § 1.97(d). Applicant requests that the IDS be placed in the file.

☒ A search report or other listing of documents from a counterpart, related, or other application dated <u>June 21, 2021</u> and having documents cited thereon is attached for the Examiner's consideration. Any of these documents not previously cited, and any additional documents are listed on the PTO/SB/08 Form.

☒ Applicant respectfully requests that the Examiner consider the listed documents and evidence that consideration by making appropriate notations on the attached form. As for any document listed on the accompanying PTO/SB/08 Form that is in a language other than English, relevance can be understood from an enclosed English abstract or corresponding English-language document or at least partial translation or from mention in the specification or in a search report for a corresponding application.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that any of the listed documents are material or constitute "prior art." If it should be determined that any of the listed documents do not constitute "prior art" under United States law, Applicant reserves the right to present to the Office the relevant facts and law regarding the appropriate status of such documents.

Applicant further reserves the right to take appropriate action to establish the patentability of the disclosed invention over the listed documents, should any of the documents be applied against the claims of the present application.

Respectfully submitted,

FAEGRE DRINKER BIDDLE & REATH LLP

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/126,366	12/18/2020	Naoki WATANABE	009628.00019\US	7560

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EXAMINER	
MCGARRY, SEAN	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
03/30/2023	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

GPD@bannerwitcoff.com
eofficeaction@bannerwitcoff.com

Notice of Abandonment	**Application No.** 17/126,366	**Applicant(s)** WATANABE et al.
	Examiner SEAN MCGARRY	**Art Unit** 1635

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☑ Applicant's failure to timely file a proper reply to the Office letter mailed on 09 September 2022.
   (a) ☐ A reply was received on ______ (with a Certificate of Mailing or Transmission dated ______), which is after the expiration of the period for reply (including a total extension of time of ______ month(s)) which expired on ______.
   (b) ☐ A proposed reply was received on ______, but it does not constitute a proper reply under 37 CFR 1.113 to the final rejection. (A proper reply under 37 CFR 1.113 to a final rejection consists only of:(1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) if this is utility or plant application, a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. Note that RCEs are not permitted in design applications.)
   (c) ☐ A reply was received on ______ but it does not constitute a proper reply, or a *bona fide* attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 8 below).
   (d) ☑ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on ______ (with a Certificate of Mailing or Transmission dated ______), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $ ______ is insufficient. A balance of $ ______ is due.
   The issue fee required by 37CFR 1.18 is $ ______ . The publication fee, if required by 37 CFR 1.18(d), is $ ______ .
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on ______ (with a Certificate of Mailing or Transmission dated ______), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record or other party authorized under 37 CFR 1.33 (b). See 37 CFR 1.138(b).

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34) upon the filing of a continuing application.

6. ☐ The decision by the Patent Trial and Appeal Board rendered on ______ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☐ The dismissal of the appeal in an application having no allowed claims. (When an appeal is dismissed in an application having no allowed claims the application stands abandoned. See MPEP 1215.04 subsections I-V for an explanation of the reasons why an appeal is dismissed.)

8. ☐ The reason(s) below:

______

/SEAN MCGARRY/
Primary Examiner, Art Unit 1635

Petitions to revive under 37 CFR 1.137, or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

EXHIBIT AN

(19) Europäisches Patentamt
European Patent Office
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(54) **ANTISENSE NUCLEIC ACID**

ANTISENSE-NUKLEINSÄURE

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WO-A1-2010/048586 WO-A1-2010/048586
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JP-A- 2002 010 790 US-A1- 2010 168 212
US-A1- 2010 168 212

- **POPPLEWELL L J ET AL: "Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials", NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB, vol. 20, no. 2, 1 February 2010 (2010-02-01), pages 102-110, XP026878306, ISSN: 0960-8966 [retrieved on 2010-01-15]**
- **POPPLEWELL L.J. ET AL.: 'Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene.' MOL. THER. vol. 17, no. 3, 2009, pages 554 - 561, XP002573471**
- **POPPLEWELL L.J. ET AL.: 'Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials.' NEUROMUSCUL. DISORD. vol. 20, no. 2, February 2010, pages 102 - 110, XP026878306**

- AARTSMA-RUS A. ET AL.: 'Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy.' NEUROMUSCUL. DISORD. vol. 12, 2002, pages S71 - S77, XP002250906
- WILTON S.D. ET AL.: 'Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript.' MOL. THER. vol. 15, no. 7, 2007, pages 1288 - 1296, XP002544728

Description

TECHNICAL FIELD

[0001] The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

[0002] Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

[0003] DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

[0004] Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

[0005] Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

[0006] The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

[0007] Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

[0008] It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057
Patent Document 2: International Publication WO 2004/048570
Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586
Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95
Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172
Non-Patent Document 3: Wilton S. D., e t al., Molecular Therapy 2007: 15: p. 1288-96
Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77
Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders , vol. 20, no. 2, p. 102-10

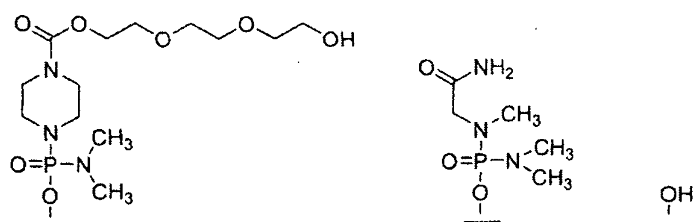
DISCLOSURE OF THE INVENTION

[0009] Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

[0010] As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

[0011] That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 36th to the 56th, from the 5' end of the 53rd exon in the human dystrophin gene.
[2] The antisense oligomer according to [1] above, which is an oligonucleotide.
[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.
[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.
[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.
[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1) (2) (3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO:11 or 35.
[10] A pharmaceutical composition comprising as an active ingredient the antisense oligomer according to any one of [1] to [9], or a pharmaceutically acceptable salt or hydrate thereof, for use in the treatment of muscular dystrophy.

[0012] The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

[0013]

FIG. 1 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. 2 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. 3 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. 4 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 5 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 6 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 7 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 8 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 9 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 10 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 11 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 12 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 13 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 14 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 15 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 16 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 17 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 18 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. 19 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

[0014] Hereinafter, **the** present **invention is** described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

1. Antisense oligomer

[0015] The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 32nd to the 56th, the 36th to the 56th, from the 5' end of the 53rd exon in the human dystrophin gene. [Exon 53 in human dystrophin gene]

[0016] In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA

precursor and mRNA. Preferably, the gene is mRNA precursor, *i.e.,* pre-mRNA.

[0017] In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, RG., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

[0018] The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

[0019] The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

[0020] Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

[0021] As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

[0022] As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, *e.g.,* SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

[0023] As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

[0024] As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 32°C. The term "moderate stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 42°C, or 5x SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42°C. The term "high stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 50°C or 0.2 x SSC, 0.1% SDS at 65°C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

[0025] When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1 % (w/v) SDS at 55°C, thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

[0026] In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

[0027] The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment

Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul SF, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score = 100 and wordlength = 12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

[0028] Examples of the nucleotide sequences complementary to the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31 st to the 57th, the 31 st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31 - 53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31 - 54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32 - 53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32 - 54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12
32 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33 - 53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33 - 54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34 - 53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34 - 54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34 - 55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35 - 53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35 - 54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35 - 55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28

(continued)

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
35 - 56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36 - 53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36 - 54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36 - 55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36 - 56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36 - 57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

[0029] Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides *(e.g.,* SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

[0030] The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript *(e.g.,* pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

[0031] Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the *in vivo* environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40°C, preferably 37°C, pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

[0032] Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell *(e.g.,* human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

[0033] The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

[0034] The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA).

[0035] The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

[0036] The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

[0037] The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines *(e.g.,* 5-methylcytosine), 5-alkyluracils *(e.g.,* 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methyl-

guanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

[0038] Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

[0039] The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, *e.g.*, LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

[0040] A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry,2008, 18, 9154-9160) (cf., *e.g.*, Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

[0041] The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, isopentyl, neopentyl, *tert*-pentyl, *n*-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

[0042] The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

[0043] The halogen includes fluorine, chlorine, bromine and iodine.

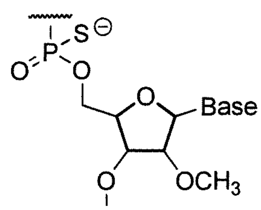
[0044] The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, *n*-propoxy, isopropoxy, *n*-butoxy, isobutoxy, *sec*-butoxy, *tert*-butoxy, *n*-pentyloxy, isopentyloxy, *n*-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

[0045] The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

[0046] The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

[0047] The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

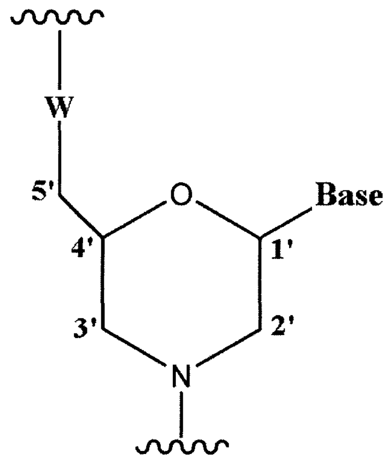
[0048] Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the -OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



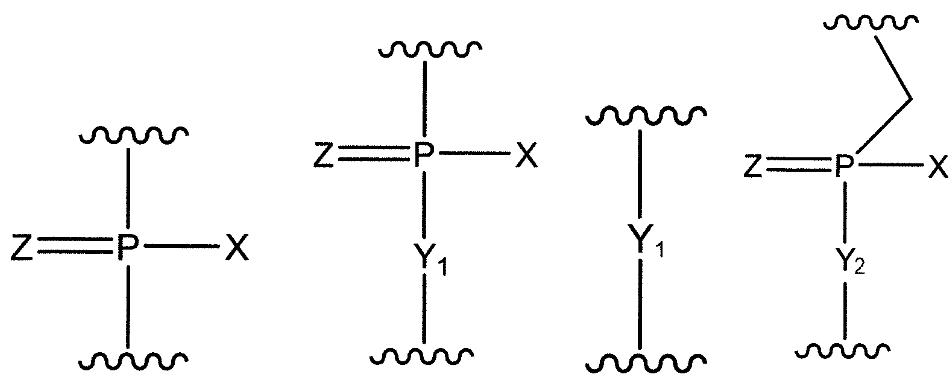
wherein Base represents a nucleobase.

[0049] The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (*e.g.*, AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (*e.g.*, Promega Inc., or Takara Co.), etc.

[0050] The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:

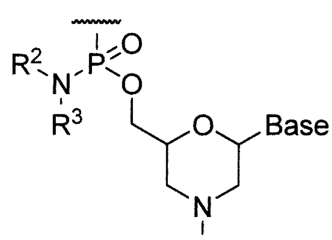


wherein Base has the same significance as defined above, and,
W represents a group shown by any one of the following groups:



wherein X represents $-CH_2R^1$, $-O-CH_2R^1$, $-S-CH_2R^1$, $-NR_2R^3$ or F;
R^1 represents H or an alkyl;
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;
Y_1 represents O, S, CH_2 or NR^1;
Y_2 represents O, S or NR^1;
Z represents O or S.

[0051] Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

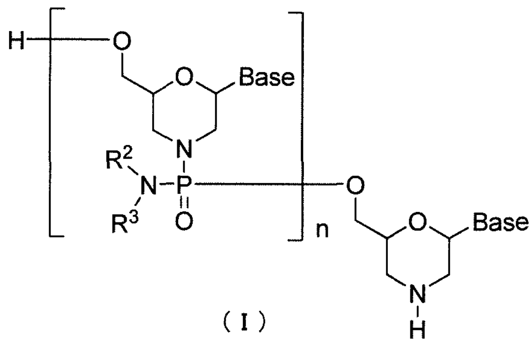


wherein Base, R^2 and R^3 have the same significance as defined above.
[0052] The morpholino oligomer may be produced in accordance with, *e.g.*, WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown

below.

[Method for producing PMO]

[0053] An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein Base, R^2 and R^3 have the same significance as defined above; and,
n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

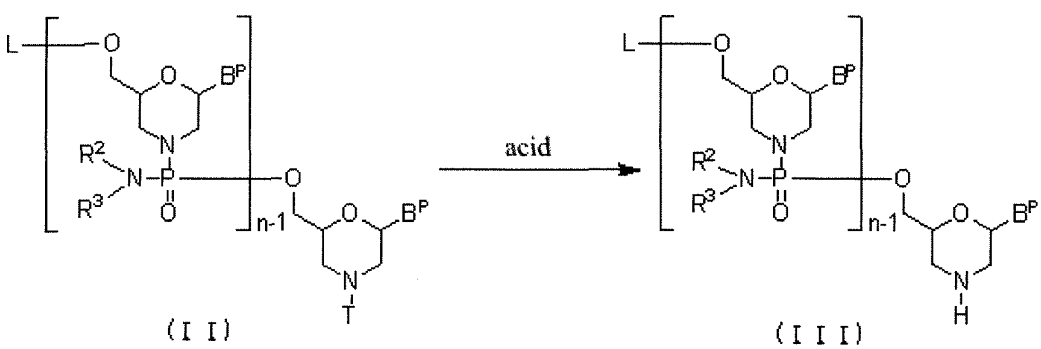
[0054] PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

[0055] The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

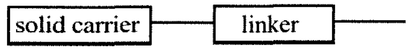
[0056] Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

[0057] The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



wherein n, R^2 and R^3 have the same significance as defined above;
each B^P independently represents a nucleobase which may optionally be protected; T represents trityl, monomethoxytrityl or dimethoxytrityl; and,
L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).



(IV)

[0058] The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

[0059] Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., *e.g.*, WO 2009/064471).

[0060] The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: *e.g.*, (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (*e.g.*, dichloromethane, acetonitrile, tetrazole, *N*-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (*e.g.*, aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin·HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene *(e.g.,* Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene *(e.g.,* NH_2-PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, *e.g.,* CPG), oxalyl-controlled pore glass (cf, *e.g.,* Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (*e.g.,* Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

[0061] A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

[0062] This step can be performed by reacting Compound (II) with an acid.

[0063] The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

[0064] An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, *e.g.,* 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

[0065] When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

[0066] The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

[0067] The reaction temperature in the reaction described above is preferably in a range of, *e.g.,* 10°C to 50°C, more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C.

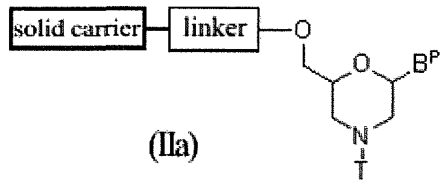
[0068] The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

[0069] After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

[0070] The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, *e.g.,* 10°C to 50°C, more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C.

[0071] The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

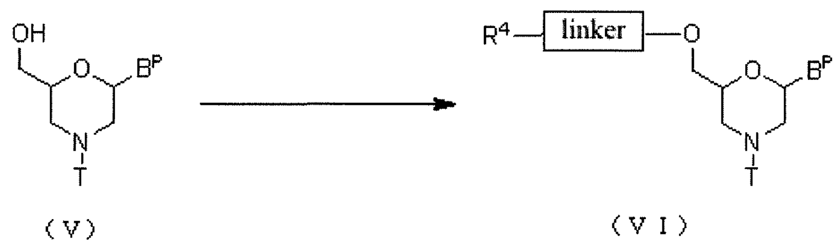
[0072] In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.



wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

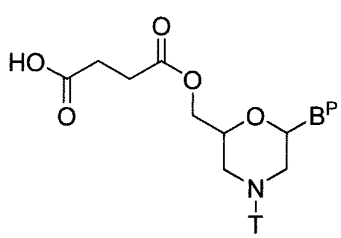
[0073] The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



wherein B^P, T and linker have the same significance as defined above; and,
R^4 represents hydroxy, a halogen or amino.

[0074] This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

[0075] In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

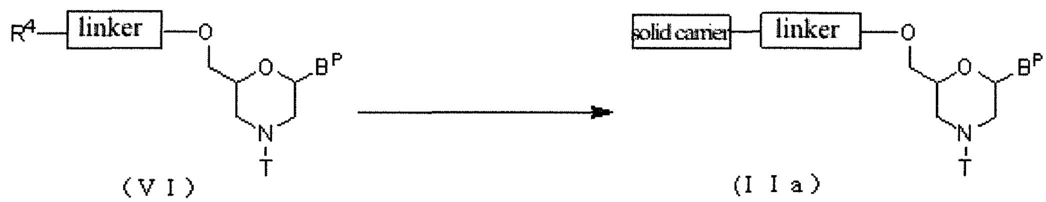


(VIa)

wherein B^P and T have the same significance as defined above.

Step 2:

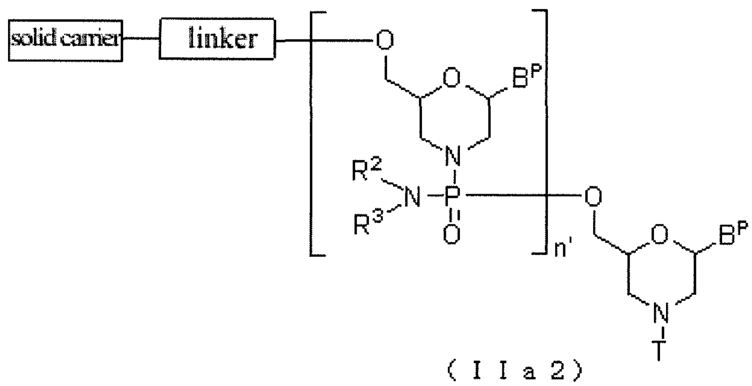
[0076] Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

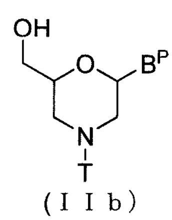
[0077] This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

[0078] In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



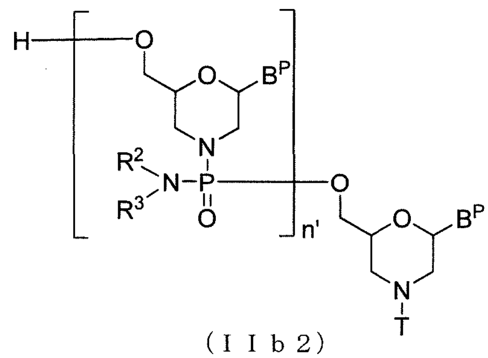
wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and, n' represents 1 to 98.

[0079] In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, *e.g.,* WO 1991/009033.



wherein B^P and T have the same significance as defined above.

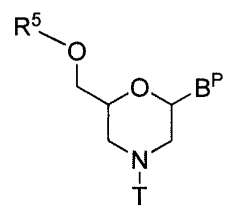
[0080] In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



(I I b 2)

wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

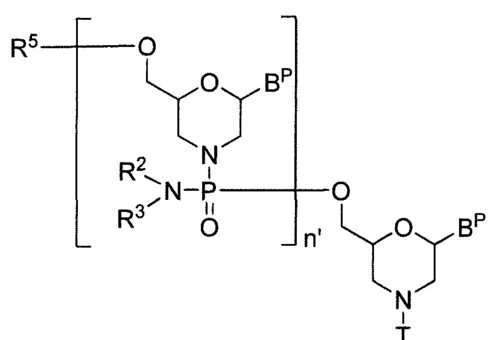
[0081] In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).



(I I c)

wherein B^P and T have the same significance as defined above; and,
R^5 represents an acyl.

[0082] In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

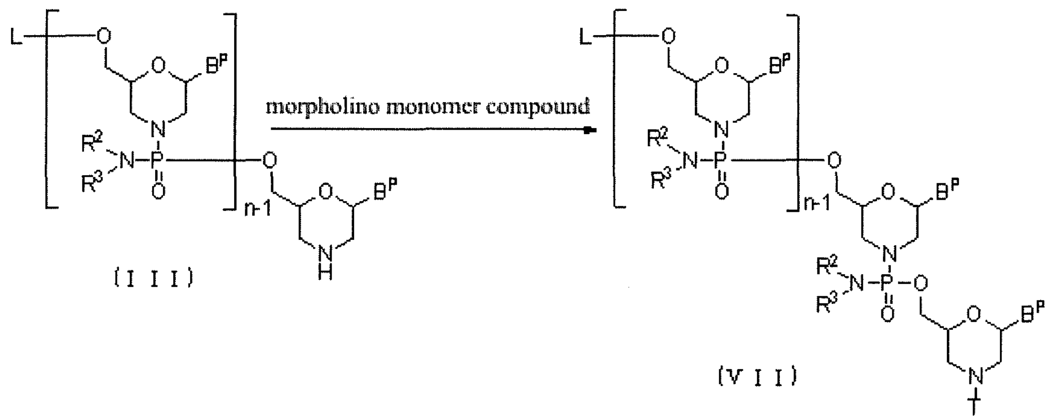


(I I c 2)

wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

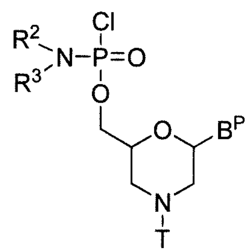
[0083] Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

[0084] This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

[0085] The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:



(VIII)

wherein B^P, R^2, R^3 and T have the same significance as defined above.

[0086] The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and *N*-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

[0087] The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, *N*-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

[0088] The reaction temperature is preferably in a range of, *e.g.,* 0°C to 100°C, and more preferably, in a range of 10°C to 50°C.

[0089] The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

[0090] Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

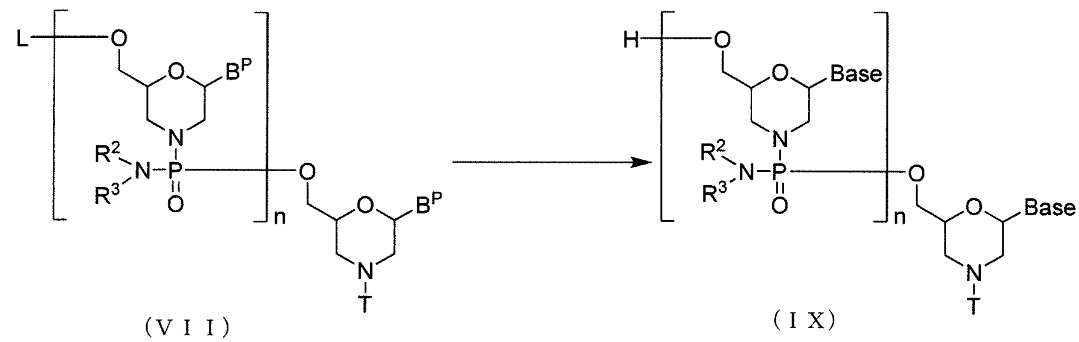
[0091] If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, *N*-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, *e.g.,* 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

[0092] The reaction temperature in this reaction is preferably in a range of 10°C to 50°C, more preferably, in a range of 10°C to 50°C, much more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C.

The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

[0093] In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

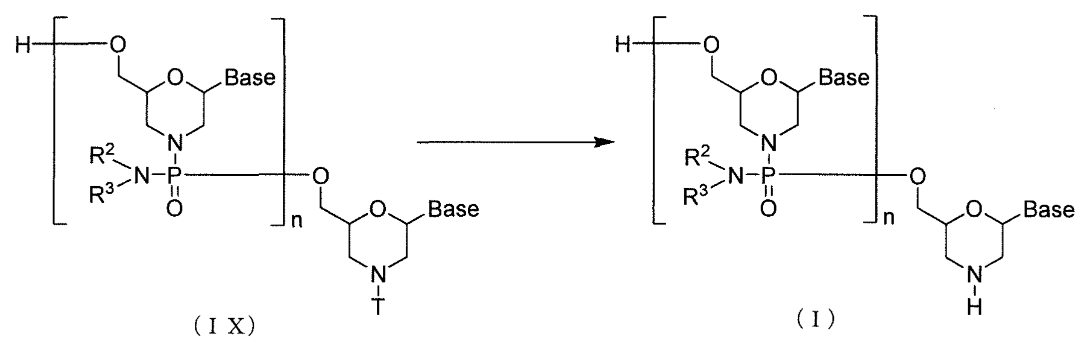
[0094] This step can be performed by reacting Compound (VII) with a deprotecting agent.

[0095] The "deprotecting agent" includes, *e.g.*, conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, *e.g.*, water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, *N*-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, *e.g.,* 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

[0096] The reaction temperature is appropriately in a range of 15°C to 75°C, preferably, in a range of 40°C to 70°C, and more preferably, in a range of 50°C to 60°C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

[0097] PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



wherein Base, n, R^2, R^3 and T have the same significance as defined above.

[0098] This step can be performed by adding an acid to Compound (IX).

[0099] The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

[0100] The reaction temperature is appropriately in a range of 10°C to 50°C, preferably, in a range of 20°C to 40°C,

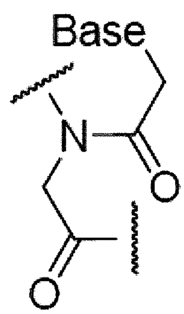
and more preferably, in a range of 25°C to 35°C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

[0101] PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., *e.g.,* WO 1991/09033).

[0102] In purification of PMO (I) using reversed phase chromatography, *e.g.*, a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

[0103] In purification of PMO (I) using ion exchange chromatography, *e.g.*, a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

[0104] A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:

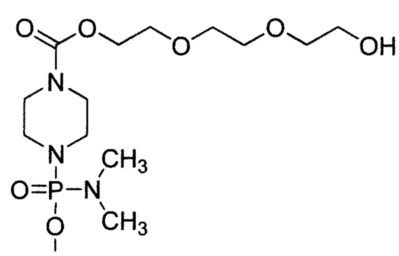


wherein Base has the same significance as defined above.

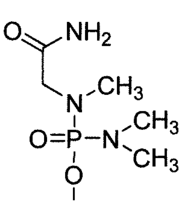
[0105] Peptide nucleic acids can be prepared by referring to, *e.g.,* the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J.Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

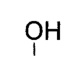
[0106] In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.



(1)



(2)



(3)

[0107] Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical composition

[0108] The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

[0109] In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

[0110] Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, *N*-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, *N*,*N'*-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, *N*-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

[0111] Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

[0112] In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

[0113] A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

[0114] In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids *(e.g.,* fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers *(e.g.,* cholesterol and phosphatidic acid), isotonizing agents *(e.g.,* sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents *(e.g.,* hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used.

The content of the additive in the composition of the present invention is appropriately 90 wt% or less, preferably 70 wt% or less and more preferably, 50 wt% or less.

[0115] The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

[0116] The composition of the present invention may be prepared into, *e.g.,* a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about -40 to -20°C, performing a primary drying at 0 to 10°C under reduced pressure, and then performing a secondary drying at about 15 to 25°C under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

[0117] The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water , physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

[0118] It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

[0119] In still another embodiment of the composition of the present disclosure there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

[0120] Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

[EXAMPLES]

[REFERENCE EXAMPLE 1]

<u>4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy} -4-oxobutanoic acid loaded onto aminomethyl polystyrene resin</u>

Step 1: Production of

4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]meth oxy}-4-oxobutanoic acid

[0121] Under argon atmosphere, 22.0 g of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimid in-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichlorometh-

ane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of

4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy} -4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

[0122] After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]meth oxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

[0123] The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV measurement

[0124]

Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

[REFERENCE EXAMPLE 2]

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpho lin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

[0125] Guanosine, 100 g, was dried at 80°C under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0°C, followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf. :Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2:

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

[0126] In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric

acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4°C overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

[0127] In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of

4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

[0128] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that *N*-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[REFERENCE EXAMPLE 3]

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

[0129] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4 (1H,3H)-dione was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[REFERENCE EXAMPLE 4]

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

[0130] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

[0131] According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31 - 55	5' end: group (3)	SEQ ID NO: 4
2	32 - 53	5' end: group (3)	SEQ ID NO: 8
3	32 - 56	5' end: group (3)	SEQ ID NO: 11
4	33 - 54	5' end: group (3)	SEQ ID NO: 15
5	34 - 58	5' end: group (3)	SEQ ID NO: 25
6	36 - 53	5' end: group (3)	SEQ ID NO: 32
7	36 - 55	5' end: group (3)	SEQ ID NO: 34
8	36 - 56	5' end: group (3)	SEQ ID NO: 35
9	36 - 57	5' end: group (3)	SEQ ID NO: 36
10	33 - 57	5' end: group (3)	SEQ ID NO: 18
11	39 - 69	Sequence corresponding to H53A(+39+69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30 - 59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32 - 56	5' end: group (1)	SEQ ID NO: 11
14	36 - 56	5' end: group (1)	SEQ ID NO: 35
15	30 - 59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23 - 47	Sequence corresponding to SEQ ID NO:429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

[EXAMPLE 1]

PMO No. 8

[0132] 4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2 -yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0

(continued)

Step	Reagent	Volume (mL)	Time (min)
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

[0133] The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2, 6-lutidine in dichloromethane.

[0134] The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55°C for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid - triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS 18 (Waters, φ50x 100 mm, 1CV=200 mL)
Flow rate	60 mL/min
Column temperature	room temperature

(continued)

Column	XTerra MS 18 (Waters, φ50x 100 mm, 1CV=200 mL)
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50% /9CV

[0135] Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

[0136] The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, φ40x 150 mm, 1CV=200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35% /15CV

[0137] Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

[0138] The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82
Found: 6923.54

[EXAMPLE 2]

PMO. No. 1

[0139] The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96
Found: 8296.24

[EXAMPLE 3]

PMO. No. 2

[0140] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13
Found: 7309.23

[EXAMPLE 4]

PMO. No. 3

[0141] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94
Found: 8270.55

[EXAMPLE 5]

PMO. No. 4

[0142] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholi n-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13
Found: 7310.17

[EXAMPLE 6]

PMO. No. 5

[0143] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholi n-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94
Found: 8270.20

[EXAMPLE 7]

PMO. No. 6

[0144] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01
Found: 5963.68

[EXAMPLE 8]

PMO. No. 7

[0145] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55
Found: 6608.85

[EXAMPLE 9]

PMO. No. 9

[0146] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11
Found: 7279.42

[EXAMPLE 10]

PMO. No. 10

[0147] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95
Found: 8295.91

[EXAMPLE 11]

PMO. No. 13

[0148] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15
Found: 7276.69

[EXAMPLE 12]

PMO. No. 14

[0149] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,1-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFEENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27

Found: 8622.29

[COMPARATIVE EXAMPLE 1]

PMO. No. 11

[0150] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63

Found: 10273.71

[COMPARATIVE EXAMPLE 2]

PMO.No.15

[0151] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33

Found: 9940.77

[COMPARATIVE EXAMPLE 3]

PMO.No.16

[0152] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94
Found: 8238.69

[TEST EXAMPLE 1]

In *vitro* assay

[0153] Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4x 10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

[0154] After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0155] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
94°C, 2 mins: thermal denaturation
[94°C, 10 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0156] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

[0157] Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase

(manufactured by Roche). The PCR program used is as follows.

94°C, 2 mins: thermal denaturation
[94°C, 15 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0158] The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0159] The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0160] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0161] The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present disclosure caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

[TEST EXAMPLE 2]

<u>In vitro assay using human fibroblasts</u>

[0162] Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

[0163] After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5x 10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

[0164] The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

[0165] Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 1 mins; 60°C, 1 mins; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 7 mins: final extension

[0166] The primers used were hEX51F and hEX55R.

hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3' (SEQ ID NO: 45)

hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

[0167] The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0168] The results are shown in FIGS. 2 and 3. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present disclosure (FIG. 2) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 2). In particular, the oligomers PMO Nos. 3 and 8 of the present disclosure exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 2).

[0169] Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present disclosure (FIG. 3) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 3). In particular, the oligomers PMO Nos. 3 and 8 of the present disclosure exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 3).

[TEST EXAMPLE 3]

<u>In vitro assay using human fibroblasts</u>

[0170] The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

[0171] After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5x 10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

[0172] The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

[0173] Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 1 mins; 60°C, 1 mins; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 7 mins: final extension

[0174] The primers used were hEx44F and h55R.

hEx44F: 5'- TGTTGAGAAATGGCGGCGT-3' (SEQ ID NO: 48)
hEx55R: 5'- TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

[0175] The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National

Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0176] The results are shown in FIGS. 4 and 5. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present disclosure caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. 4) or deletion of exons 48-52 (FIG. 5). Also, the oligomers PMO Nos. 3 and 8 of the present disclosure were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. 4).

[TEST EXAMPLE 4]

Western blotting

[0177] The oligomer PMO No. 8 of the present disclosure was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

[0178] The oligomer PMO No. 3 or 8 of the present disclosure was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental results

[0179] The results are shown in FIGS. 6 and 7. In this experiment it was confirmed by western blotting (FIG. 6) and immunostaining (FIG. 7) that the oligomers PMO Nos. 3 and 8 of the present disclosure induced expression of the dystrophin protein.

[TEST EXAMPLE 5]

In vitro assay using human fibroblasts

[0180] The experiment was performed as in TEST EXAMPLE 3.

Experimental results

[0181] The results are shown in FIG. 8. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present disclosure caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present disclosure (FIG. 8).

[TEST EXAMPLE 6]

In vitro assay

[0182] Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53 61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
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H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
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H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
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H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
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Antisense oligomer	Nucleotide sequence	SEQ ID NO:
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H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

[0183] RD cells (human rhabdomyosarcoma cell line) were plated at 3x 10^5 in a 6-well plate and cultured in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37°C and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

[0184] After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0185] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
94°C, 2 mins: thermal denaturation
[94°C, 10 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0186] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0187] Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94°C, 2 mins: thermal denaturation
[94°C, 15 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0188] The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

[0189] The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0190] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0191] The results are shown in FIGS. 9 to 17. These experiments revealed that, when the antisense oligomers were designed at position 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

[TEST EXAMPLE 7]

[0192] Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5x 10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

[0193] After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0194] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows..

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 30 seconds; 60°C, 30 seconds; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 10 mins: final extension

[0195] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0196] The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0197] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0198] The results are shown in FIGS. 18 and 19. These experiments revealed that the oligomer PMO No. 8 of the present disclosure caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. 18). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present disclosure caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present disclosure (FIG. 19). These results showed that the sequences with -OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

[0199] Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are

the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

[0200] Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

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Claims

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

2. The antisense oligomer according to claim 1, which is an oligonucleotide.

3. The antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

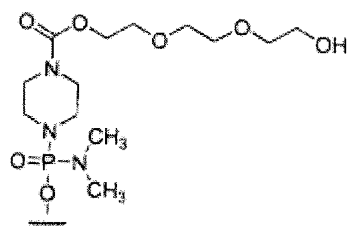
4. The antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

5. The antisense oligomer according to claim 3 or 4, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

6. The antisense oligomer according to claim 1, which is a morpholino oligomer.

7. The antisense oligomer according to claim 6, which is a phosphorodiamidate morpholino oligomer.

8. The antisense oligomer according to claim 6 or 7, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



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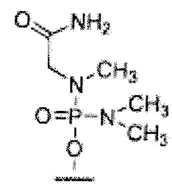


(2)

(3)

9. The antisense oligomer according to any one of claims 1 to 8, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

10. A pharmaceutical composition comprising as an active ingredient the antisense oligomer according to any one of claims 1 to 9, or a pharmaceutically acceptable salt or hydrate thereof, for use in the treatment of muscular dystrophy.

11. The pharmaceutical composition for use of claim 10, which is administered for the treatment of Duchenne muscular dystrophy.

Patentansprüche

1. Antisense-Oligomer, welches das Überspringen des 53. Exons im menschlichen Dystrophingen verursacht, bestehend aus einer Nucleotidsequenz, die zu irgendeiner der Sequenzen, bestehend aus den 32. bis 56. oder den 36. bis 56. Nucleotiden vom 5'-Ende des 53. Exons im menschlichen Dystrophingen, komplementär ist.

2. Antisense-Oligomer nach Anspruch 1, welches ein Oligonucleotid ist.

3. Antisense-Oligomer nach Anspruch 2, wobei die Zuckerkomponente und/oder die Phosphat bindende Region mindestens eines Nucleotids, das das Oligonucleotid bildet, modifiziert ist.

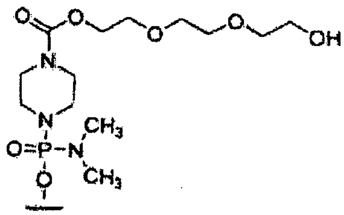
4. Antisense-Oligomer nach Anspruch 3, wobei die Zuckerkomponente mindestens eines Nucleotids, das das Oligonucleotid bildet, eine Ribose ist, bei der die 2'-OH-Gruppe durch irgendeines, ausgewählt aus der Gruppe, bestehend aus OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br und I (wobei R ein Alkyl oder ein Aryl ist und R' ein Alkylen ist), ersetzt ist.

5. Antisense-Oligomer nach Anspruch 3 oder 4, wobei die Phosphat bindende Region mindestens eines Nucleotids, das das Oligonucleotid bildet, irgendeines, ausgewählt aus der Gruppe, bestehend aus einer Phosphorothioatbindung, einer Phosphorodithioatbindung, einer Alkylphosphonatbindung, einer Phosphoramidatbindung und einer Boranophosphatbindung, ist.

6. Antisense-Oligomer nach Anspruch 1, welches ein Morpholinooligomer ist.

7. Antisense-Oligomer nach Anspruch 6, welches ein Phosphorodiamidatmorpholinooligomer ist.

8. Antisense-Oligomer nach Anspruch 6 oder 7, wobei das 5'-Ende irgendeines der Gruppen der nachstehenden chemischem Formeln (1) bis (3) ist:



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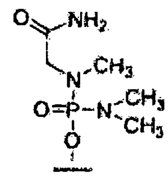

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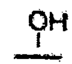


(3)

9. Antisense-Oligomer nach einem der Ansprüche 1 bis 8, bestehend aus der durch SEQ ID-Nr.: 11 oder 35 gezeigten Nucleotidsequenz.

10. Pharmazeutische Zusammensetzung, umfassend als einen Wirkstoff das Antisense-Oligomer nach einem der Ansprüche 1 bis 9 oder ein pharmazeutisch akzeptables Salz oder Hydrat davon, zur Verwendung bei der Behandlung von Muskeldystrophie.

11. Pharmazeutische Zusammensetzung zur Verwendung nach Anspruch 10, welche zur Behandlung von Duchenne-Muskeldystrophie verabreicht wird.

Revendications

1. Oligomère antisens qui provoque l'omission du 53^{e} exon dans le gène de dystrophine humaine, constitué d'une séquence de nucléotides complémentaire à l'une quelconque des séquences constituées des 32^{e} au 56^{e} ou des 36^{e} au 56^{e} nucléotides à partir de l'extrémité 5' du 53^{e} exon dans le gène de dystrophine humaine.

2. Oligomère antisens selon la revendication 1, qui est un oligonucléotide.

3. Oligomère antisens selon la revendication 2, dans lequel le fragment sucre et/ou la région de liaison de phosphate d'au moins un nucléotide constituant l'oligonucléotide sont modifiés.

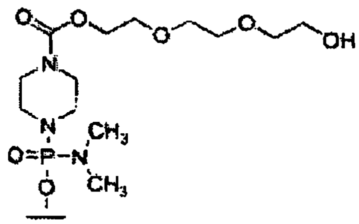
4. Oligomère antisens selon la revendication 3, dans lequel le fragment sucre d'au moins un nucléotide constituant l'oligonucléotide est un ribose dans lequel le groupe 2'-OH est remplacé n'importe quel groupe choisi dans le groupe constitué de OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br et I (où R est un alkyle ou un aryle et R' est un alkylène).

5. Oligomère antisens selon la revendication 3 ou 4, dans lequel la région de liaison de phosphate d'au moins un nucléotide constituant l'oligonucléotide est n'importe quel groupe choisi dans le groupe constitué d'une liaison phosphorothioate, une liaison phosphorodithioate, une liaison alkylphosphonate, une liaison phosphoramidate et une liaison boranophosphate.

6. Oligomère antisens selon la revendication 1, qui est un oligomère morpholino.

7. Oligomère antisens selon la revendication 6, qui est un oligomère phosphorodiamidate morpholino.

8. Oligomère antisens selon la revendication 6 ou 7, dans lequel l'extrémité 5' est n'importe lequel des groupes des formules chimiques (1) à (3) ci-dessous :



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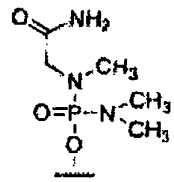

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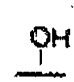


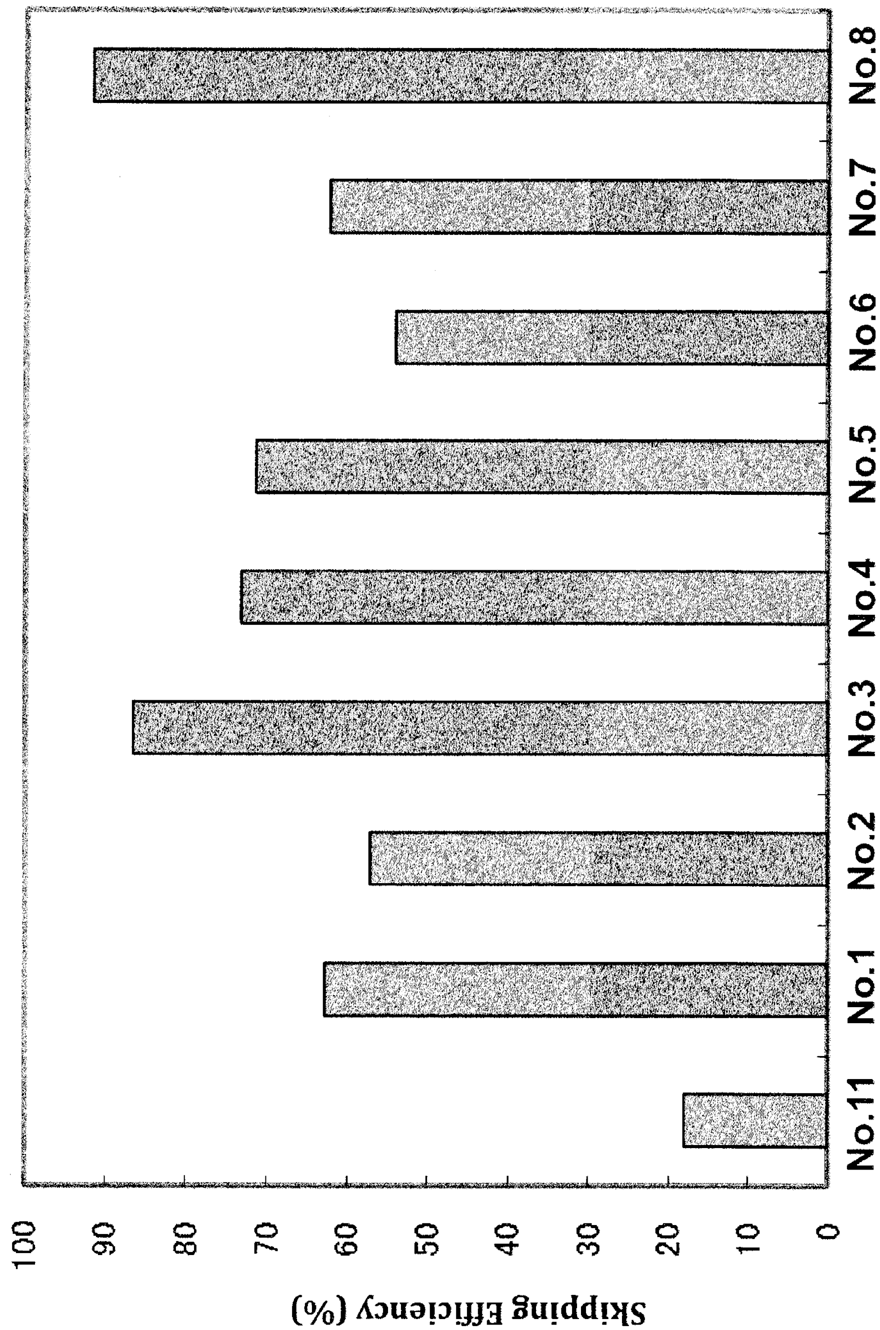
(3)

9. Oligomère antisens selon l'une quelconque des revendications 1 à 8, constitué de la séquence de nucléotides représentée par SEQ ID No. : 11 ou 35.

10. Composition pharmaceutique comprenant, en tant que principe actif, l'oligomère antisens selon l'une quelconque des revendications 1 à 9, ou un sel ou hydrate de celui-ci, acceptable sur le plan pharmaceutique, utilisable pour le traitement de la dystrophie musculaire.

11. Composition pharmaceutique utilisable selon la revendication 10, qui est administrée pour le traitement de la dystrophie musculaire de Duchenne.

Figure 1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Skipping Efficiency (%)

Figure 2

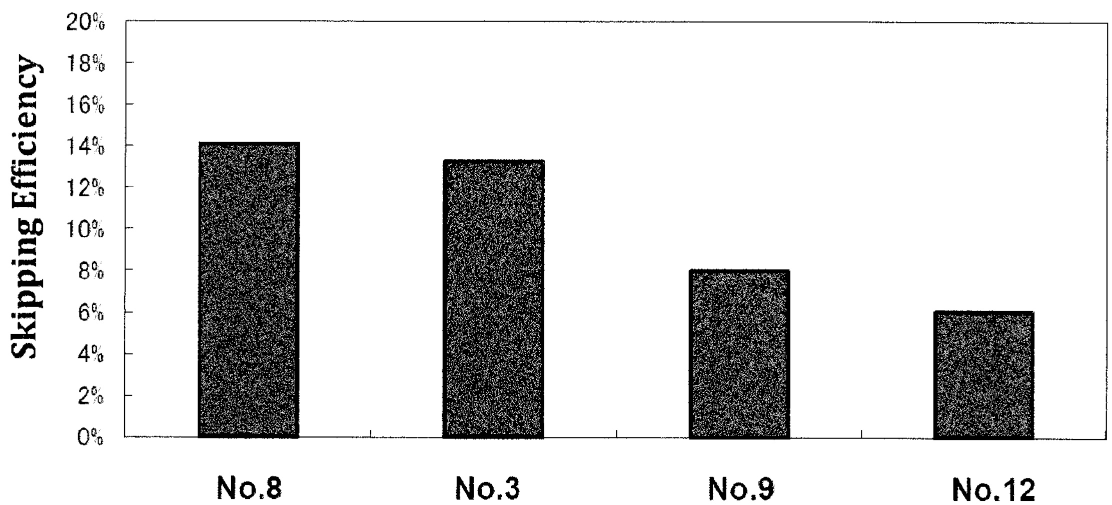


Figure 3

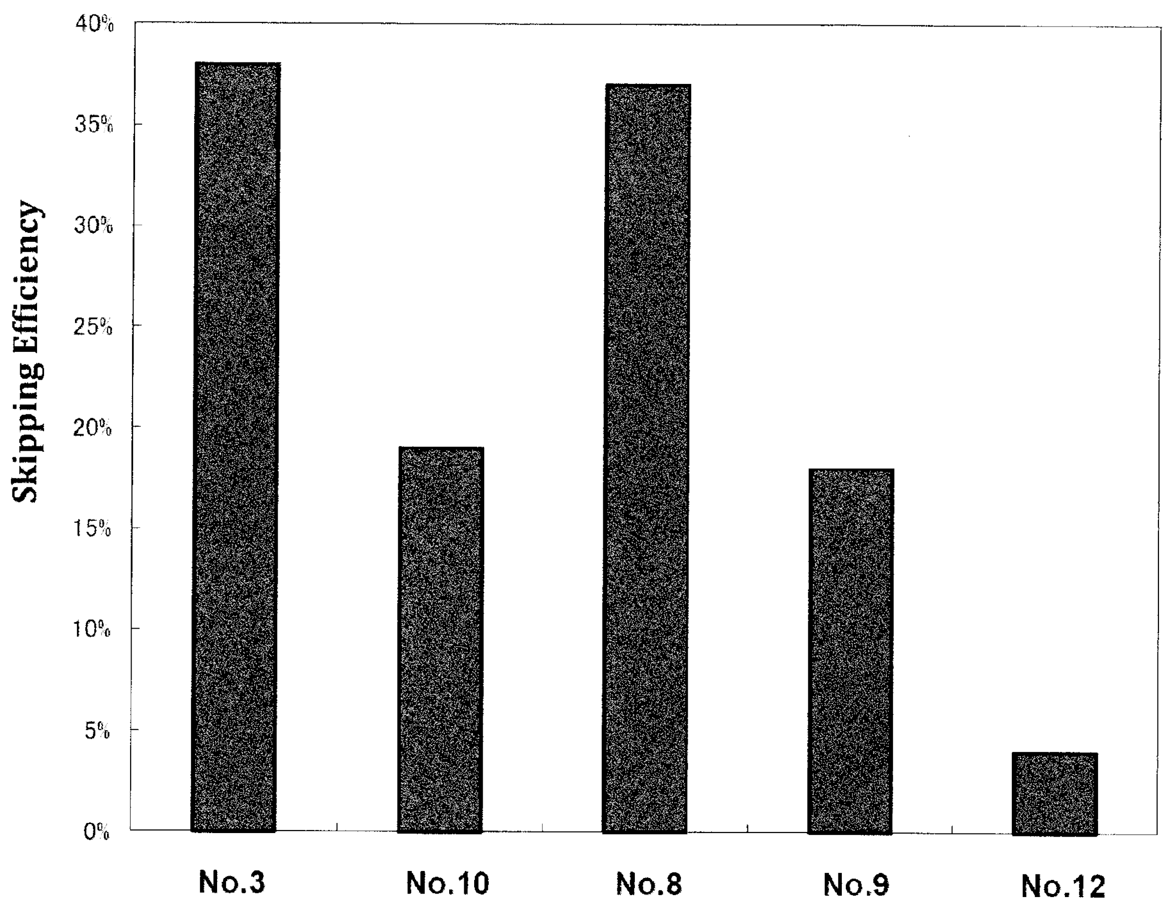

Skipping Efficiency
40%
35%
30%
25%
20%
15%
10%
5%
0%
No.3
No.10
No.8
No.9
No.12

Figure 4

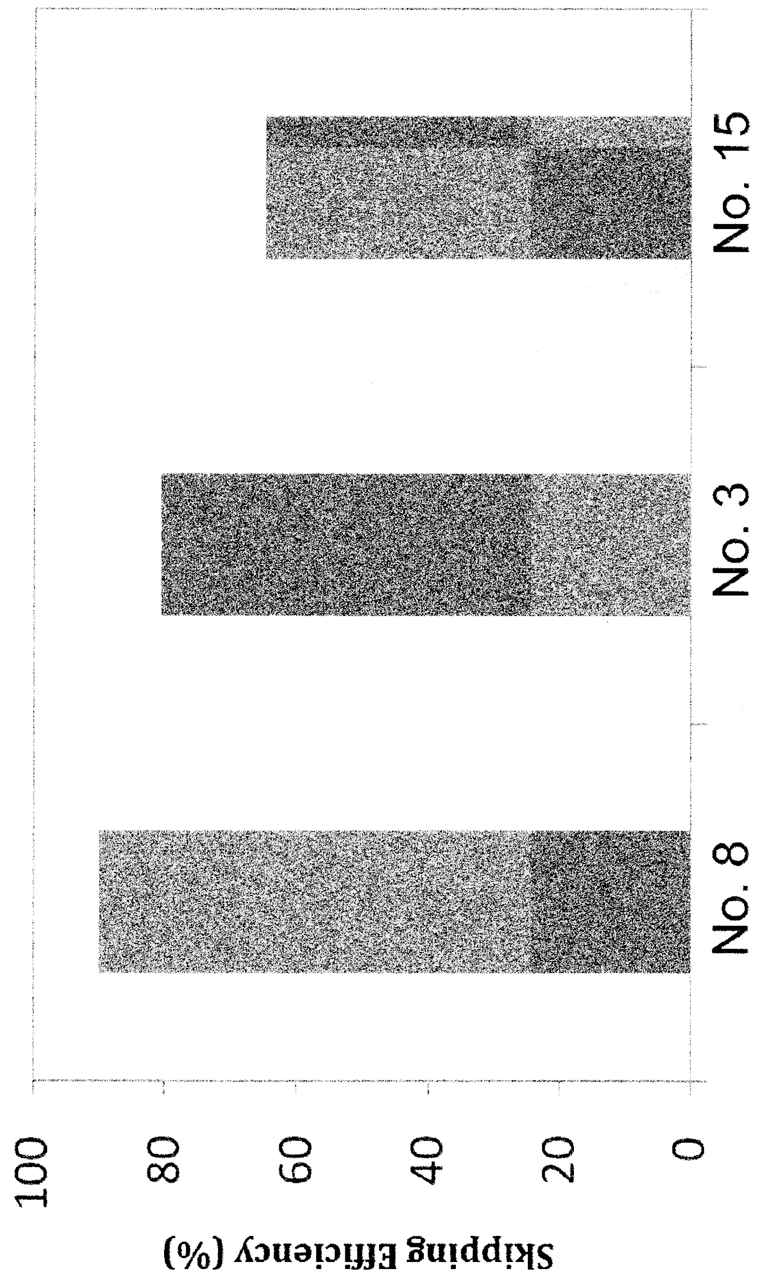


Figure 5

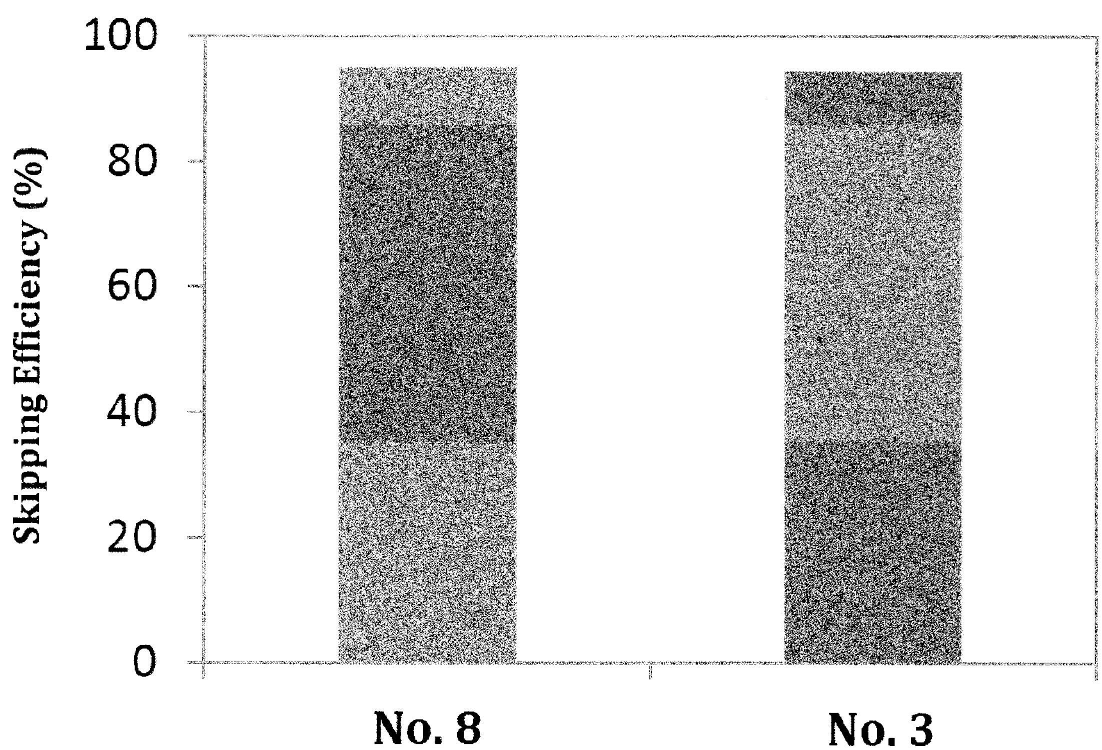


Figure 6

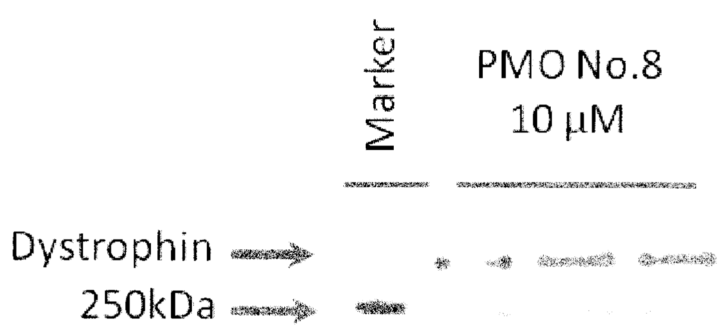


Figure 7

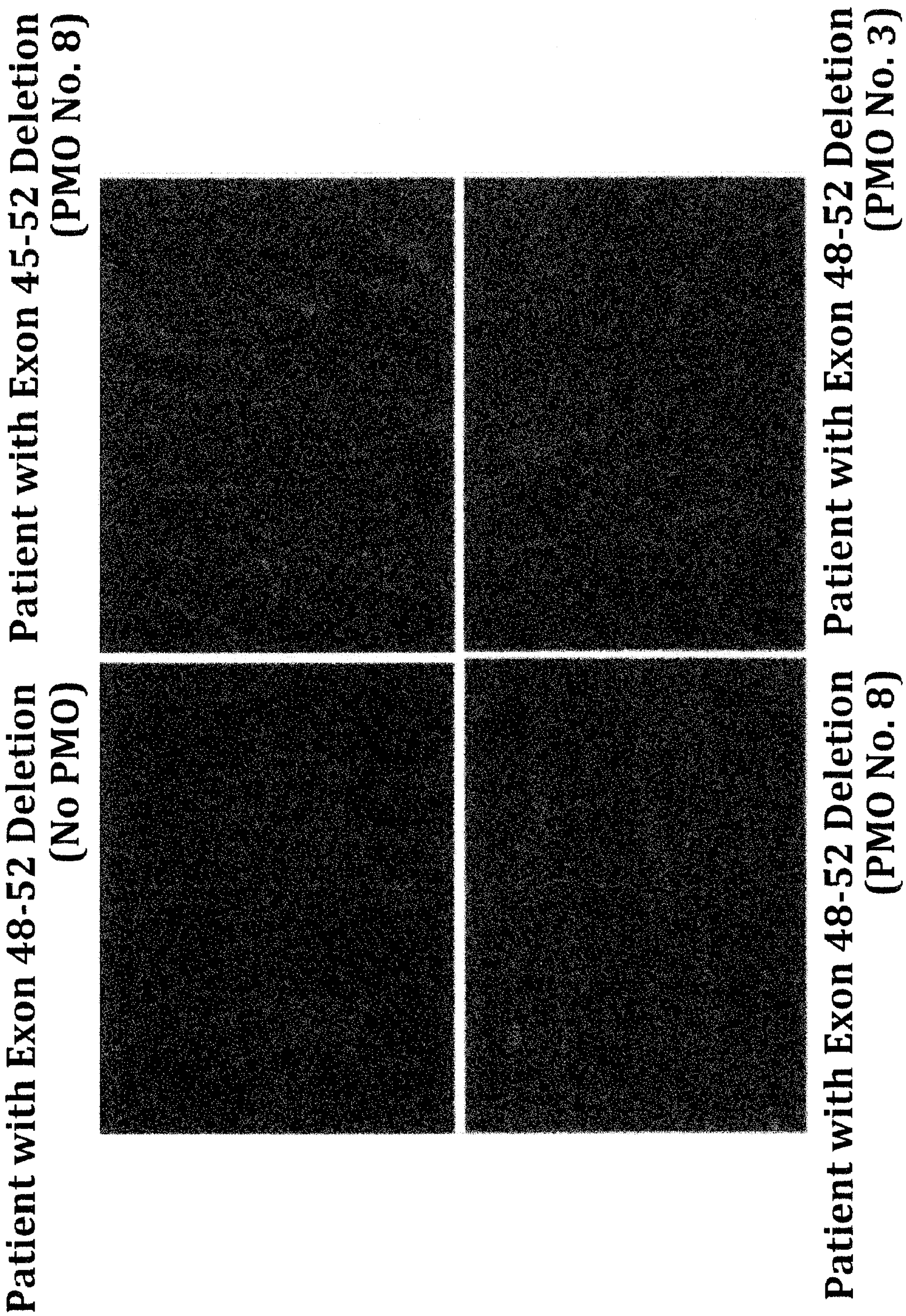

Patient with Exon 45-52 Deletion
(PMO No. 8)
Patient with Exon 48-52 Deletion
(PMO No. 3)
Patient with Exon 48-52 Deletion
(No PMO)
Patient with Exon 48-52 Deletion
(PMO No. 8)

Figure 8

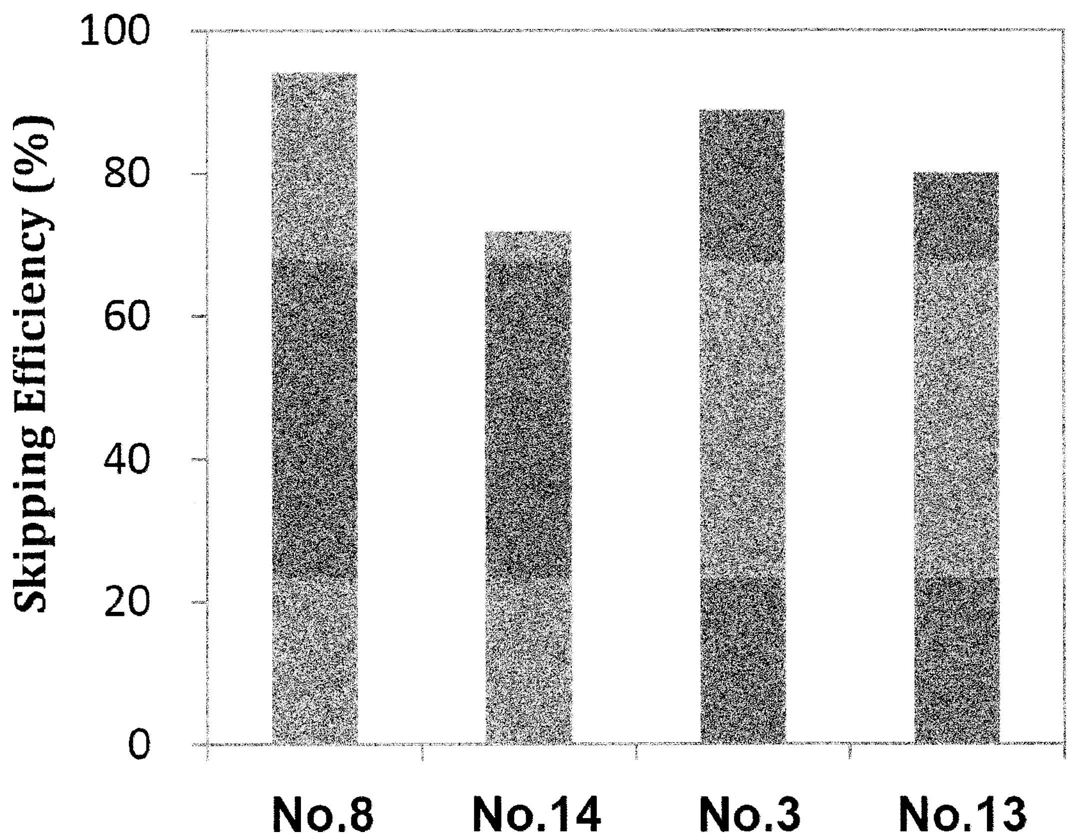


Figure 9

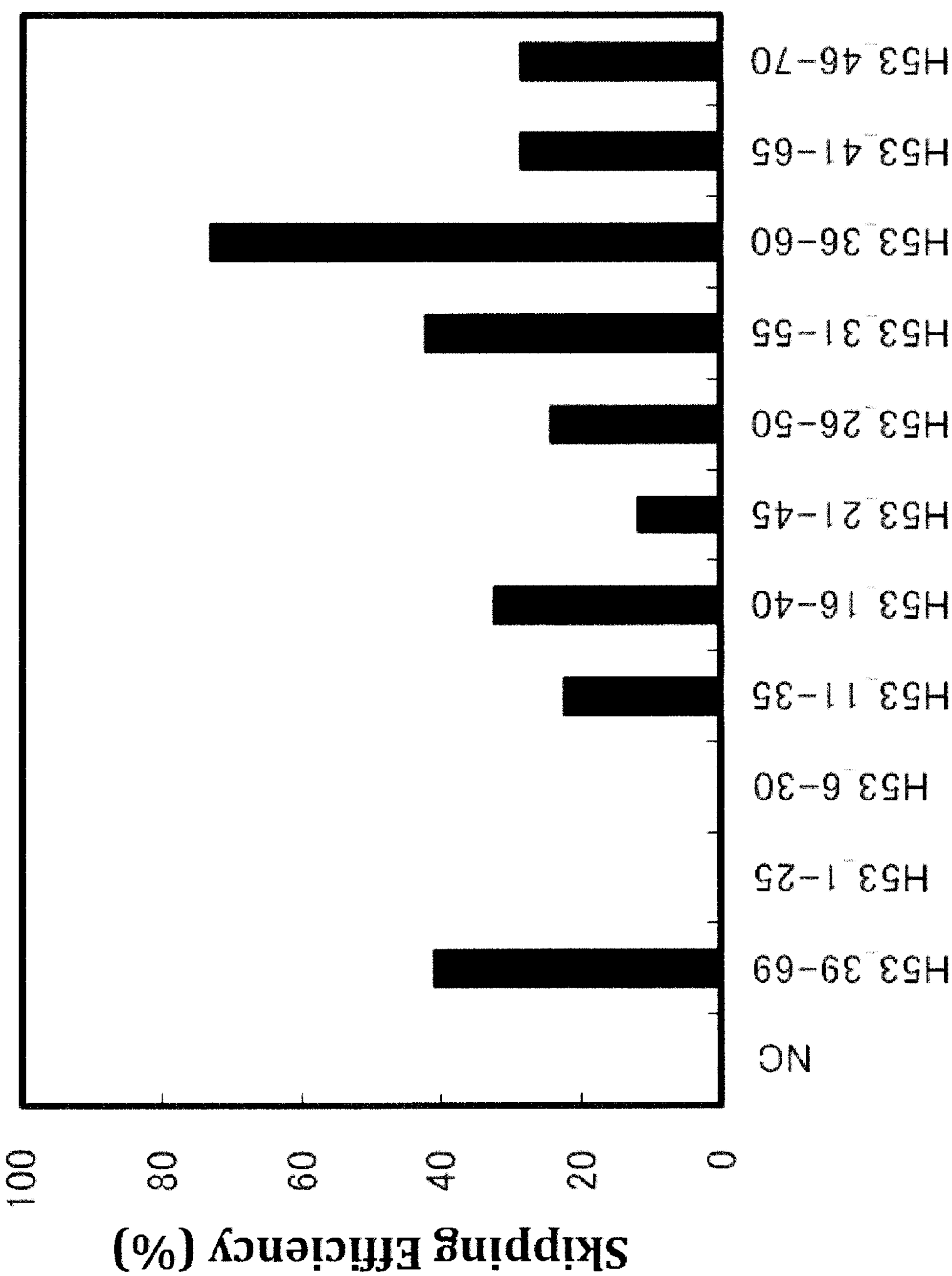

H53_46-70
H53_41-65
H53_36-60
H53_31-55
H53_26-50
H53_21-45
H53_16-40
H53_11-35
H53_6-30
H53_1-25
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 10

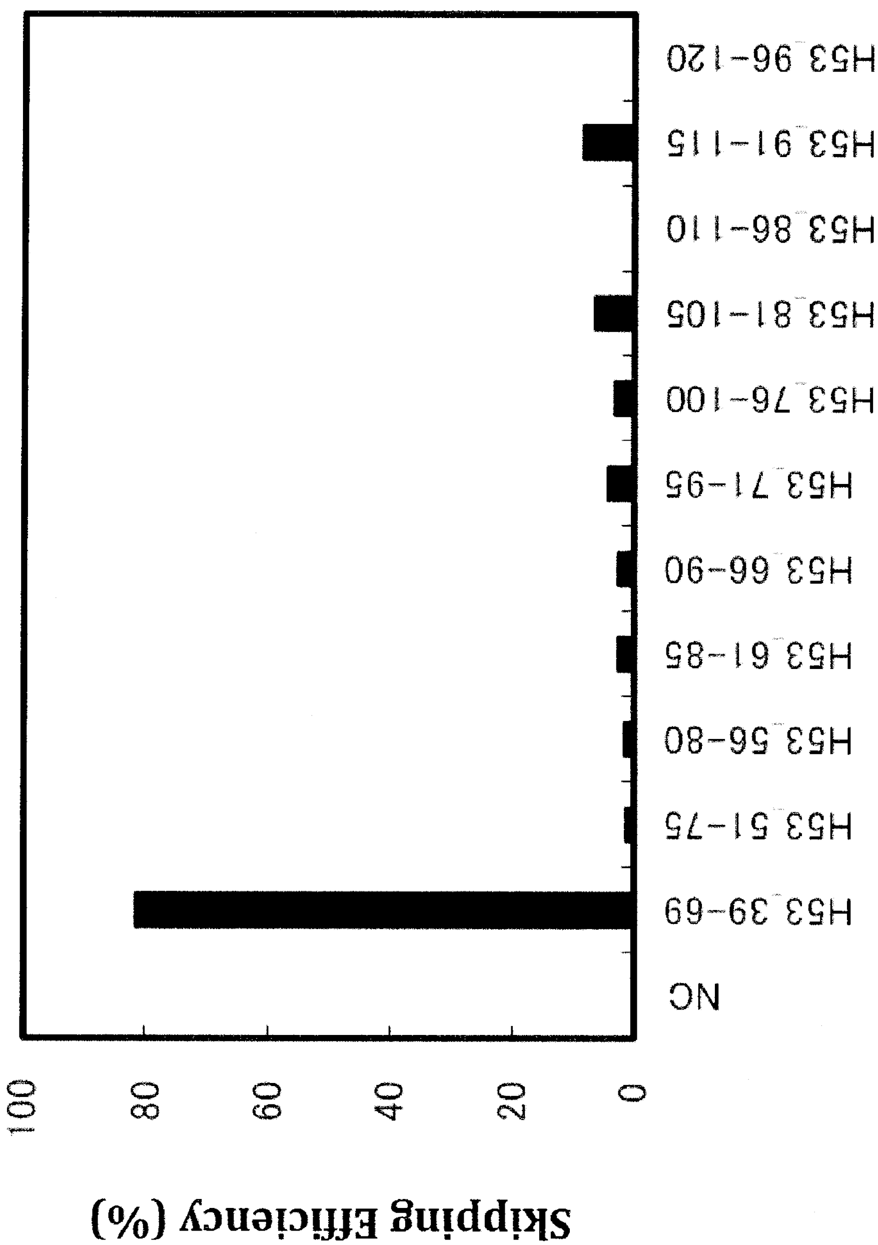

H53 96-120
H53 91-115
H53 86-110
H53 81-105
H53 76-100
H53 71-95
H53 66-90
H53 61-85
H53 56-80
H53 51-75
H53 39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 11

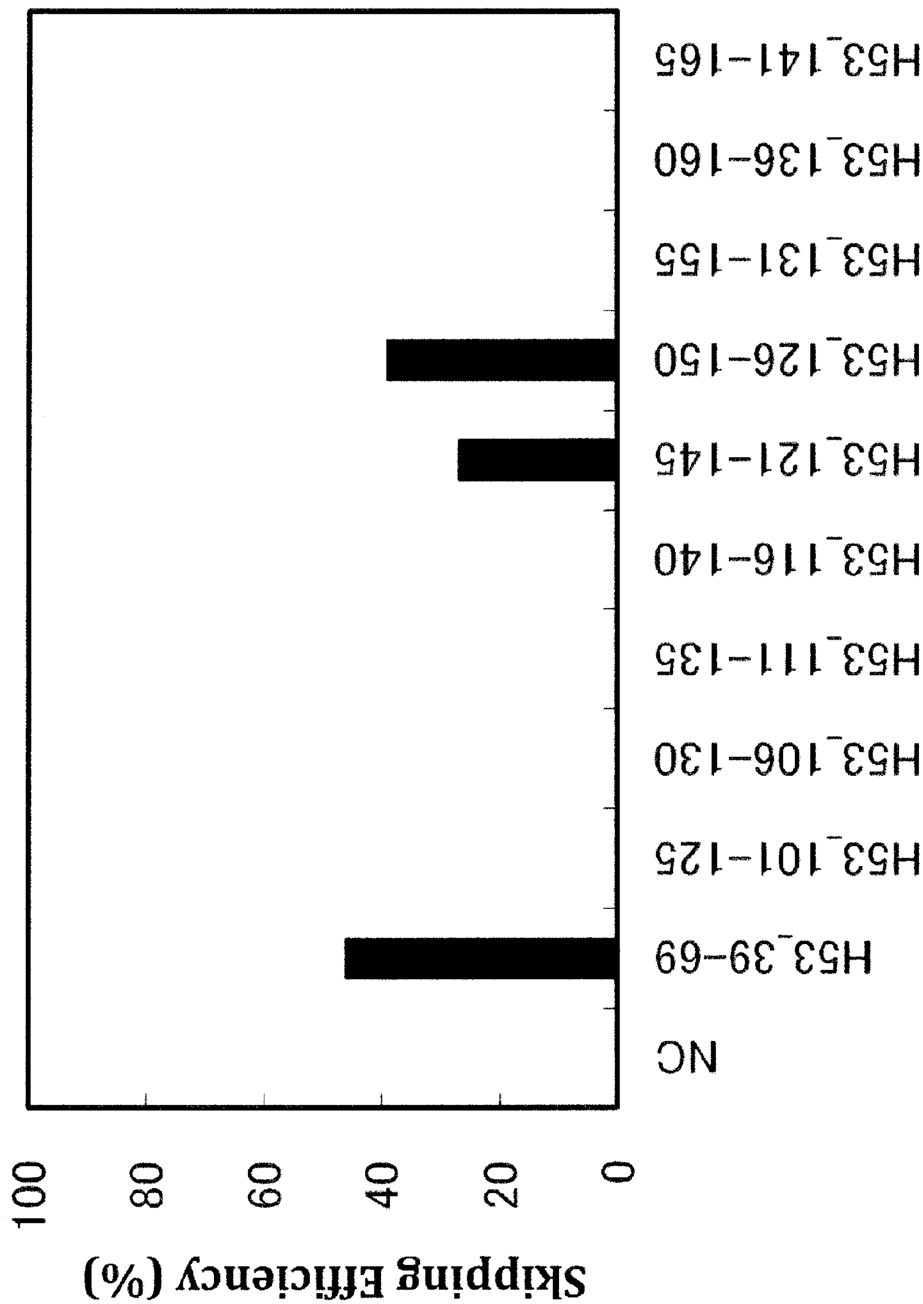

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_101-125
H53_106-130
H53_111-135
H53_116-140
H53_121-145
H53_126-150
H53_131-155
H53_136-160
H53_141-165

Figure 12

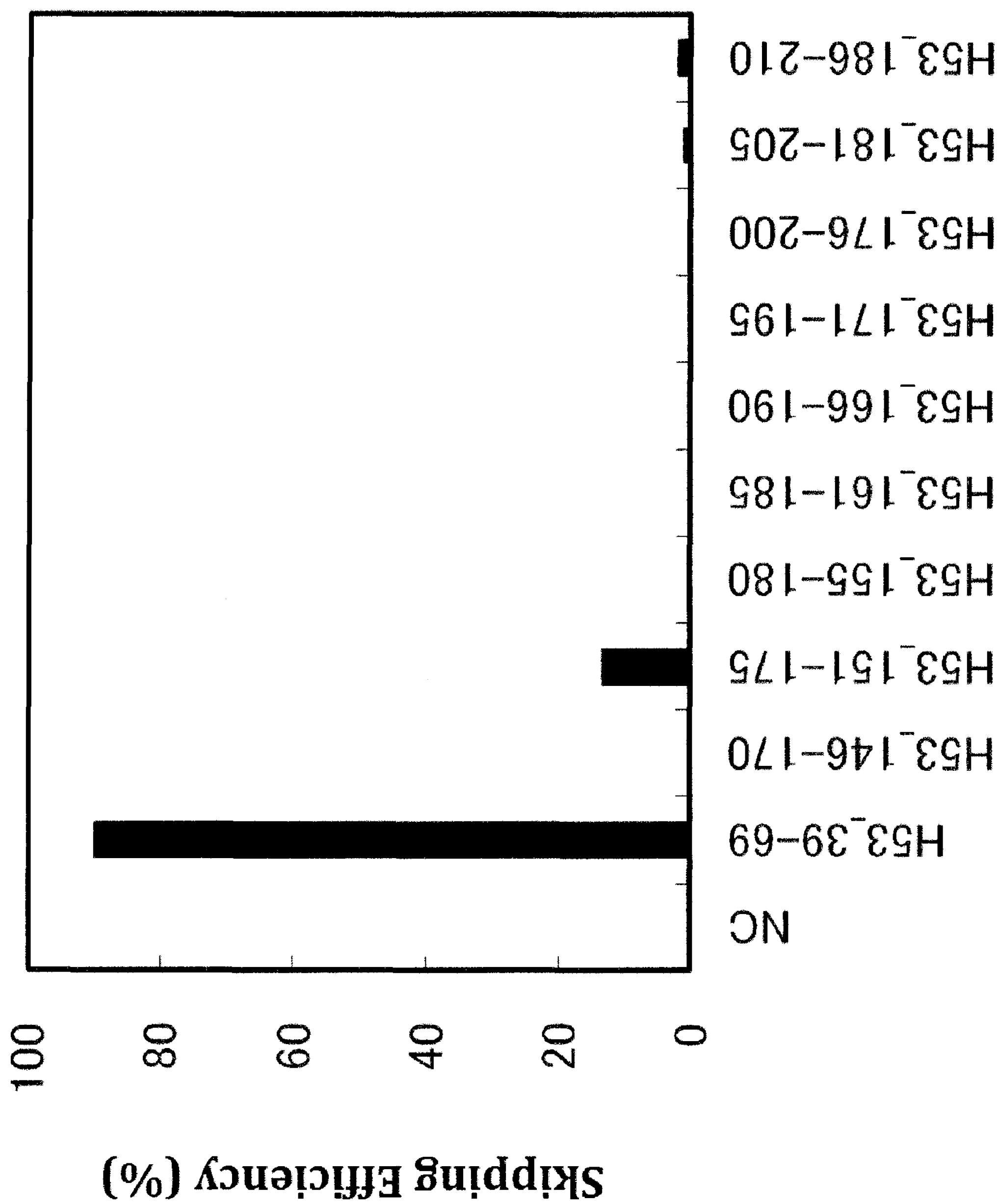

H53_186-210
H53_181-205
H53_176-200
H53_171-195
H53_166-190
H53_161-185
H53_155-180
H53_151-175
H53_146-170
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 13

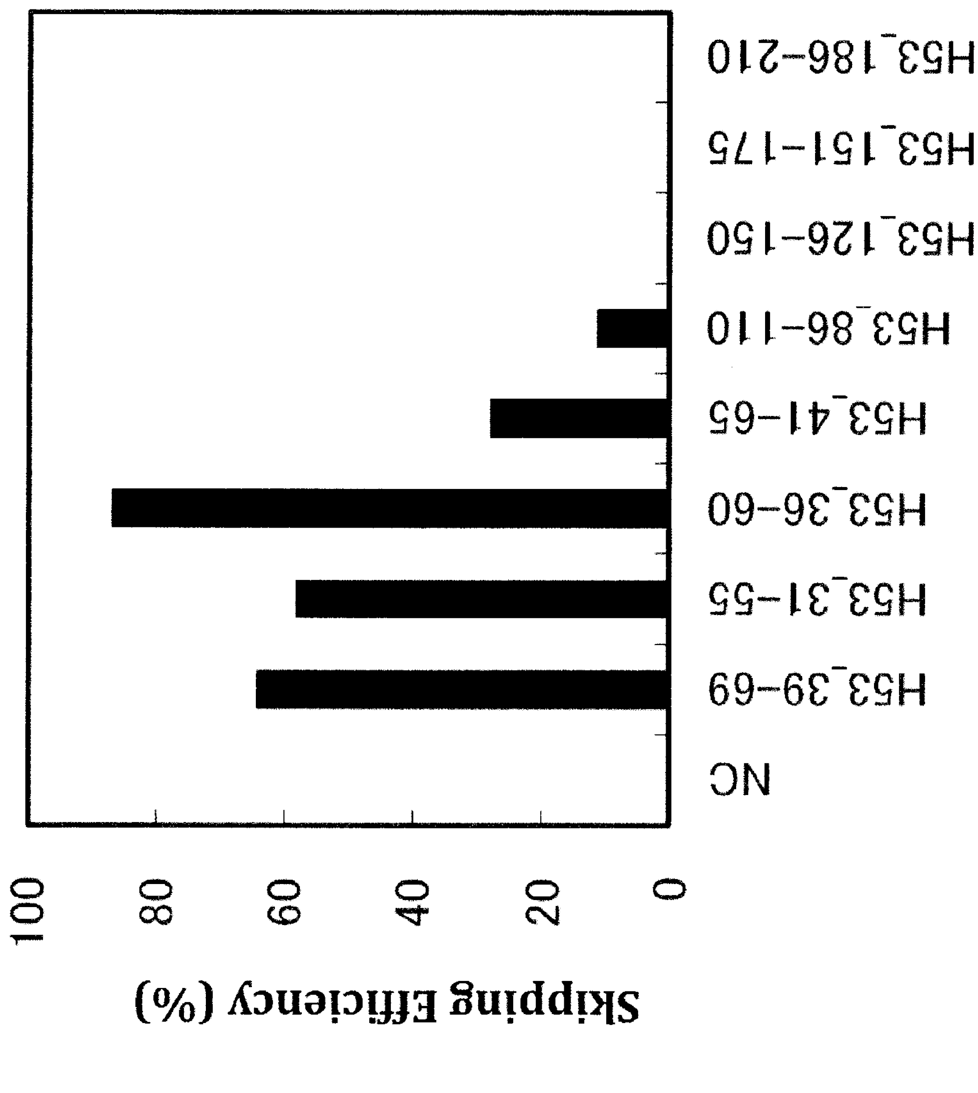
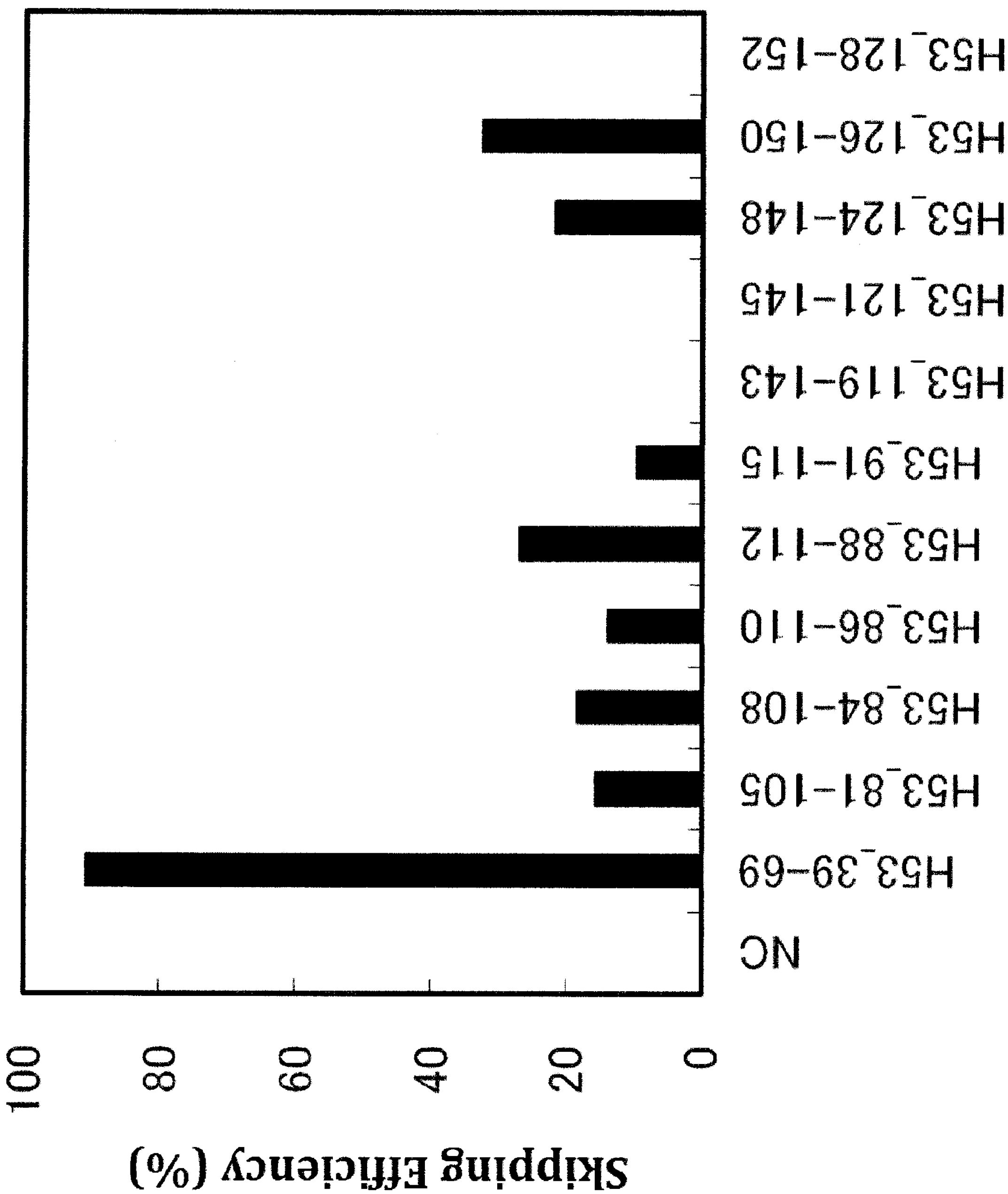

H53_186-210
H53_151-175
H53_126-150
H53_86-110
H53_41-65
H53_36-60
H53_31-55
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 14



NC
H53_39-69
H53_81-105
H53_84-108
H53_86-110
H53_88-112
H53_91-115
H53_119-143
H53_121-145
H53_124-148
H53_126-150
H53_128-152
0
20
40
60
80
100
Skipping Efficiency (%)

Figure 15

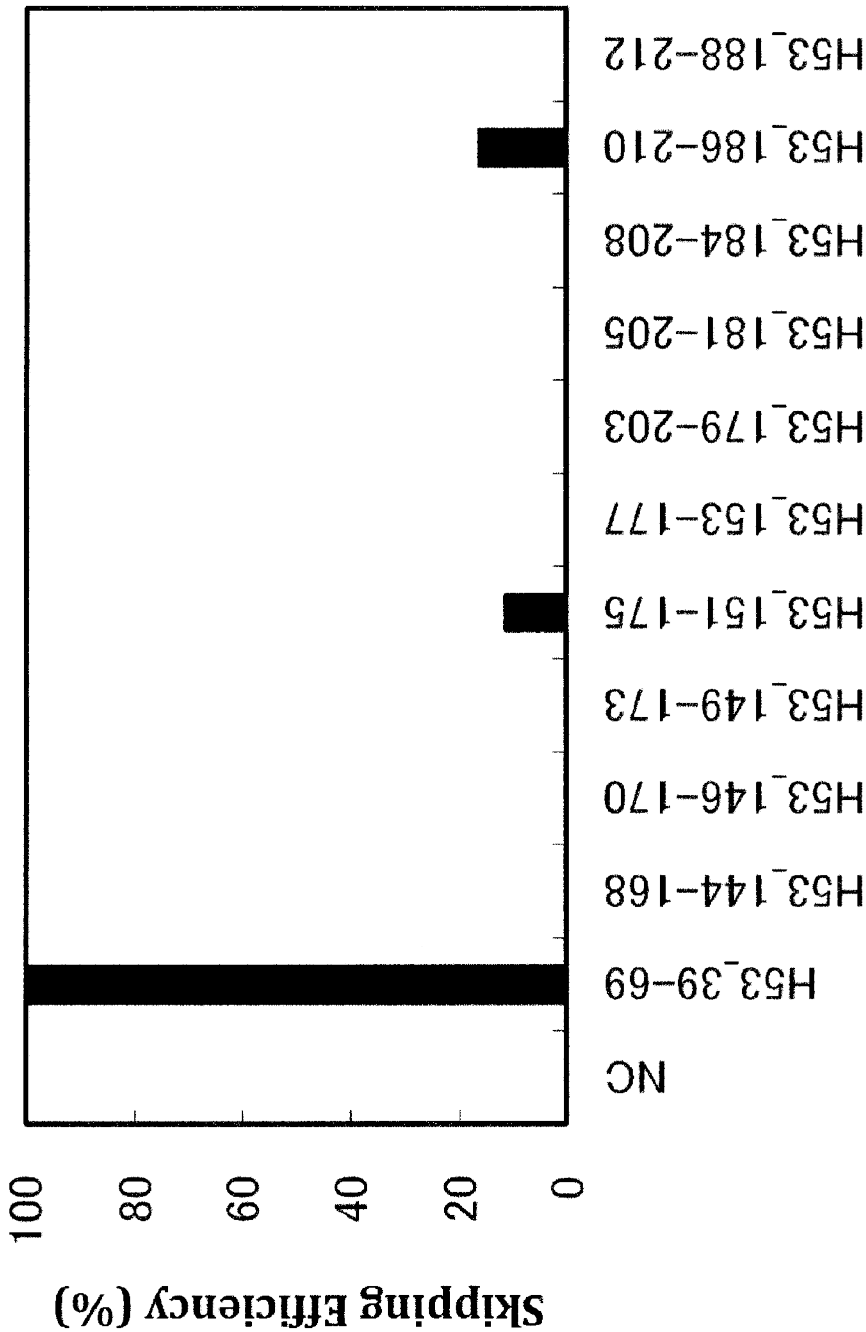

H53_188-212
H53_186-210
H53_184-208
H53_181-205
H53_179-203
H53_153-177
H53_151-175
H53_149-173
H53_146-170
H53_144-168
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 16

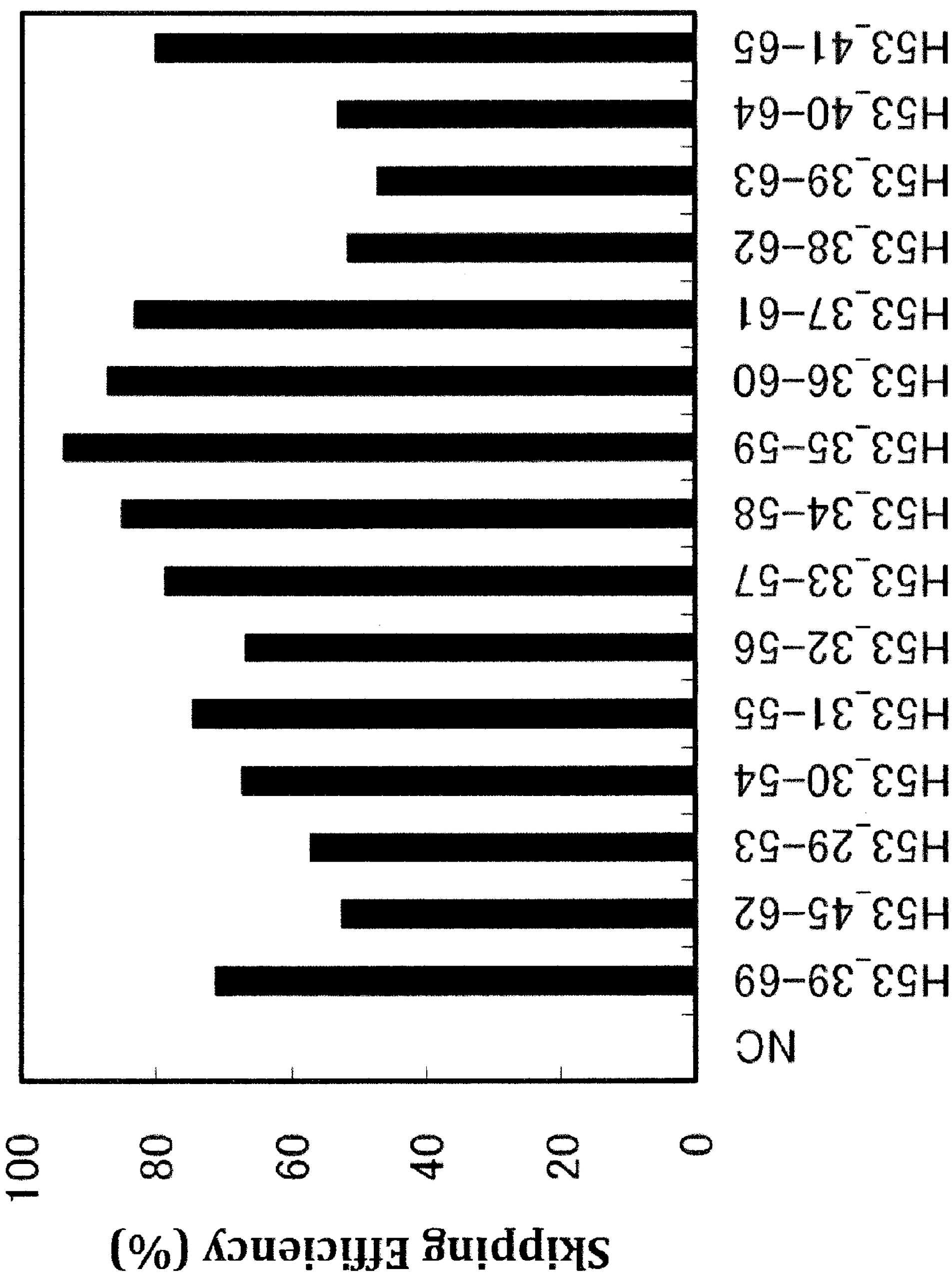
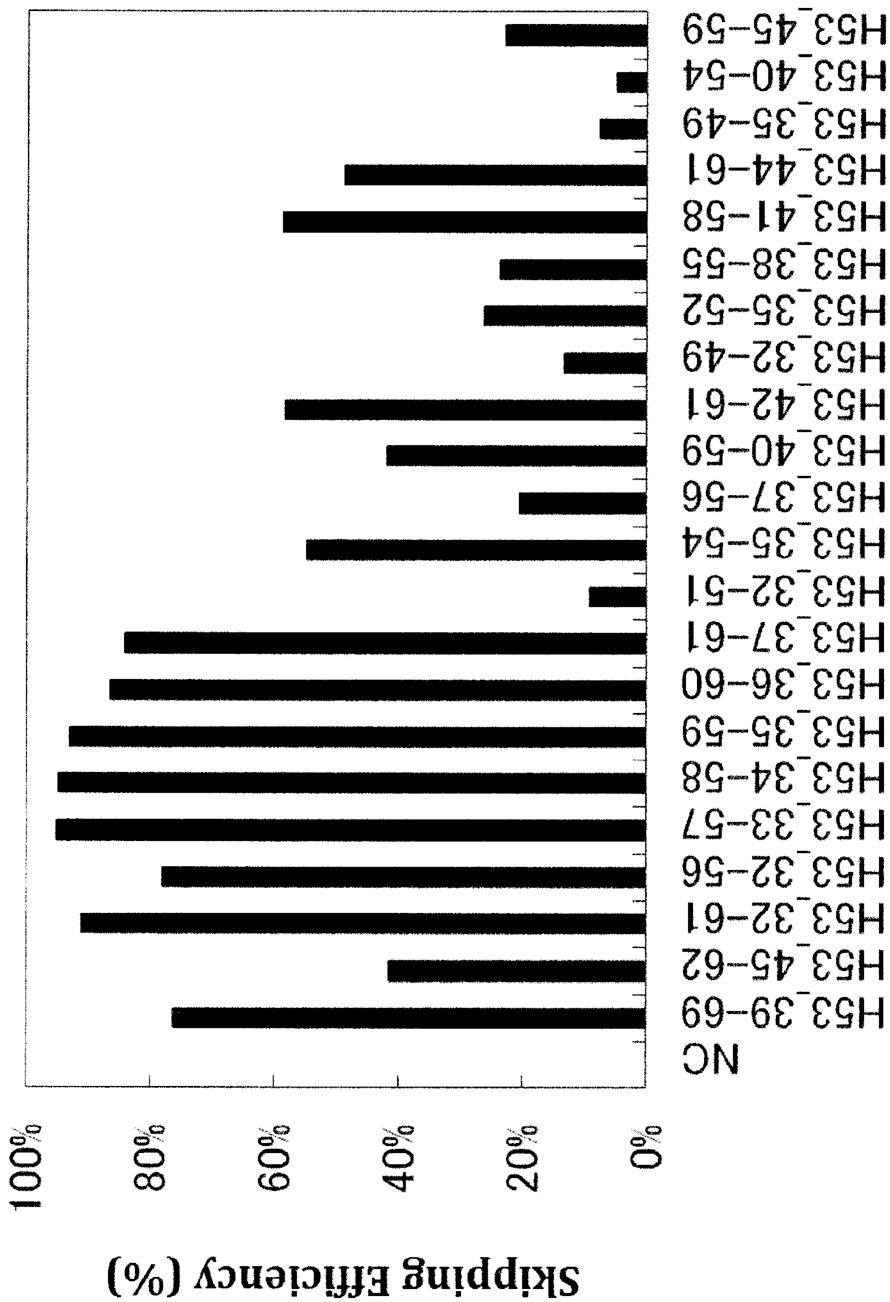
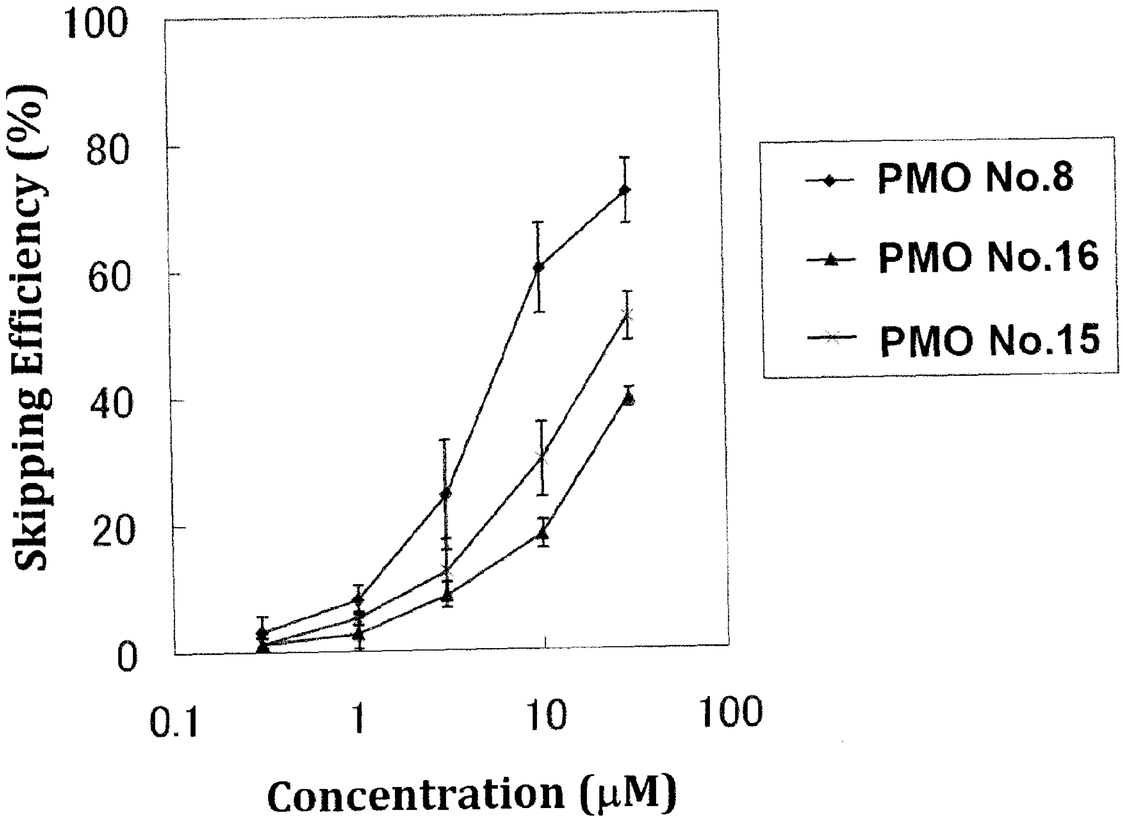

Skipping Efficiency (%)
100
80
60
40
20
0
H53_41-65
H53_40-64
H53_39-63
H53_38-62
H53_37-61
H53_36-60
H53_35-59
H53_34-58
H53_33-57
H53_32-56
H53_31-55
H53_30-54
H53_29-53
H53_45-62
H53_39-69
NC

Figure 17



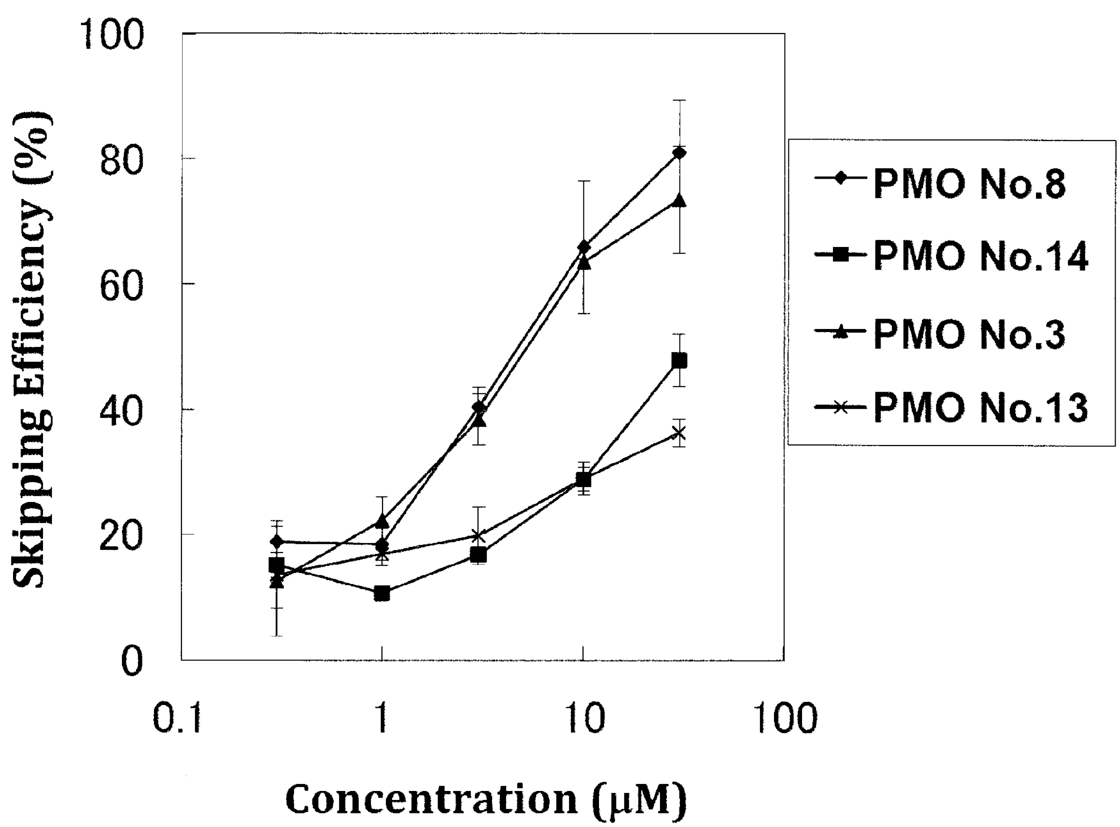
Skipping Efficiency (%)
0%
20%
40%
60%
80%
100%
NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
H53_35-52
H53_38-55
H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59

Figure 18



100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 19


Skipping Efficiency (%)
100
80
60
40
20
0
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

REFERENCES CITED IN THE DESCRIPTION

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EXHIBIT AO

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European Patent Office
Office européen des brevets

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(54) **ANTISENSE NUCLEIC ACIDS**

ANTISENSE-NUKLEINSÄUREN

ACIDES NUCLÉIQUES ANTISENS

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Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

EP 3 018 211 B1

Description

TECHNICAL FIELD

[0001] The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

[0002] Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

[0003] DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

[0004] Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

[0005] Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

[0006] The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

[0007] Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

[0008] It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4;

Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057
Patent Document 2: International Publication WO 2004/048570
Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586
Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95
Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172
Non-Patent Document 3: Wilton S. D., e t al., Molecular Therapy 2007: 15: p. 1288-96
Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77
Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders , vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

[0009] Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

[0010] As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

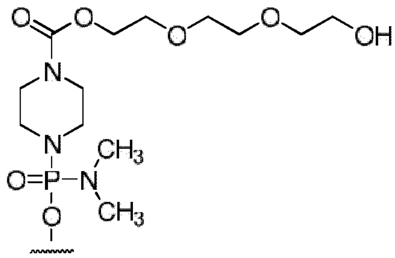
[0011] That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.
[2] The antisense oligomer according to [1] above, which is an oligonucleotide.
[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I, wherein R is an alkyl or an aryl and R' is an alkylene.

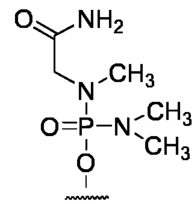
[0012] The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

[0013] Also disclosed herein are:

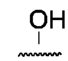
[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.
[2] The antisense oligomer according to [1] above, which is an oligonucleotide.
[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.
[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.
[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.
[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)



(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

BRIEF DESCRIPTION OF DRAWINGS

[0014]

FIG. 1 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. 2 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. 3 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. 4 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 5 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 6 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 7 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 8 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 9 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 10 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 11 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 12 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 13 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhab-

domyosarcoma cells (RD cells).

FIG. 14 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 15 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 16 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 17 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 18 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. 19 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

[0015] Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

[0016] All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed September 1, 2010, from which the priority was claimed.

1. Antisense oligomer

[0017] The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of <u>the 36th to the 60th nucleotides</u> from the 5' end of the 53rd exon in the human dystrophin gene.

[0018] Also disclosed herein is an antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in human dystrophin gene]

[0019] In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, *i.e.*, pre-mRNA.

[0020] In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, RG., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

[0021] The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

[0022] The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

[0023] Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

[0024] As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

[0025] As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, *e.g.*, SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

[0026] As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

[0027] As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 32°C. The term "moderate stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 42°C, or 5x SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42°C. The term "high stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 50°C or 0.2 x SSC, 0.1% SDS at 65°C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

[0028] When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55°C, thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (*e.g.*, a PCR Labeling Mix (Roche Diagnostics), etc.).

[0029] In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

[0030] The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul SF, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score = 100 and wordlength = 12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

[0031] Examples of the nucleotide sequences complementary to the sequences consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31 - 53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31 - 54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32 - 53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32 - 54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3 '	SEQ ID NO: 12
32 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33 - 53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33 - 54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33 - 55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34 - 53	5'-CCGGTTCTGAAGGTGTTCTT-3 '	SEQ ID NO: 20
34 - 54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34 - 55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34 - 56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34 - 57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35 - 53	5 '-CCGGTTCTGAAGGTGTTCT-3 '	SEQ ID NO: 26
35 - 54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35 - 55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35 - 56	5 '-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35 - 57	5 '-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36 - 53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36 - 54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36 - 55	5'-CTCCGGTTCTGAAGGTGTTC-3 '	SEQ ID NO: 34
36 - 56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36 - 57	5 '-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36 - 58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

[0032] It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to

any one of the sequences consisting of the 36th to the 60th nucleotides (*e.g.*, SEQ ID NO: 57), from the 5' end of the 53rd exon in the human dystrophin gene.

[0033] Also disclosed herein is an oligomer consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (*e.g.*, SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

[0034] Also disclosed herein is an oligomer consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (*e.g.*, SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

[0035] The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (*e.g.*, pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

[0036] Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

[0037] Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the *in vivo* environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40°C, preferably 37°C, pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

[0038] Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (*e.g.*, human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

[0039] The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

[0040] The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

[0041] The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

[0042] The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

[0043] The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (*e.g.*, 5-methylcytosine), 5-alkyluracils (*e.g.*, 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

[0044] Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or 1, wherein R represents an alkyl or an aryl and R' represents an alkylene.

[0045] The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, *e.g.*, LNA (locked nucleic acid) or

ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

[0046] A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry ,2008, 18, 9154-9160) (cf., *e.g.*, Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

[0047] The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, isopentyl, neopentyl, *tert*-pentyl, *n*-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

[0048] The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

[0049] The halogen includes fluorine, chlorine, bromine and iodine.

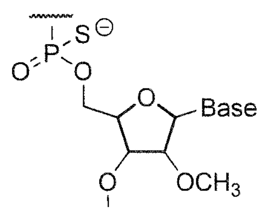
[0050] The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, *n*-propoxy, isopropoxy, *n*-butoxy, isobutoxy, *sec*-butoxy, *tert*-butoxy, *n*-pentyloxy, isopentyloxy, *n*-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

[0051] The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

[0052] The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

[0053] The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

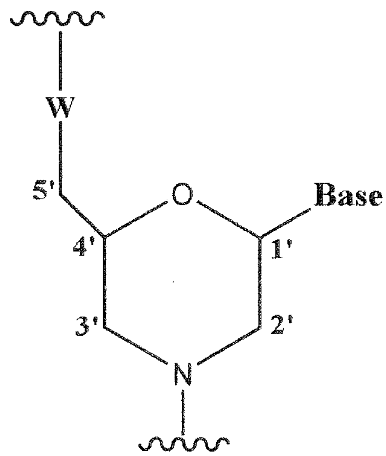
[0054] Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the -OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



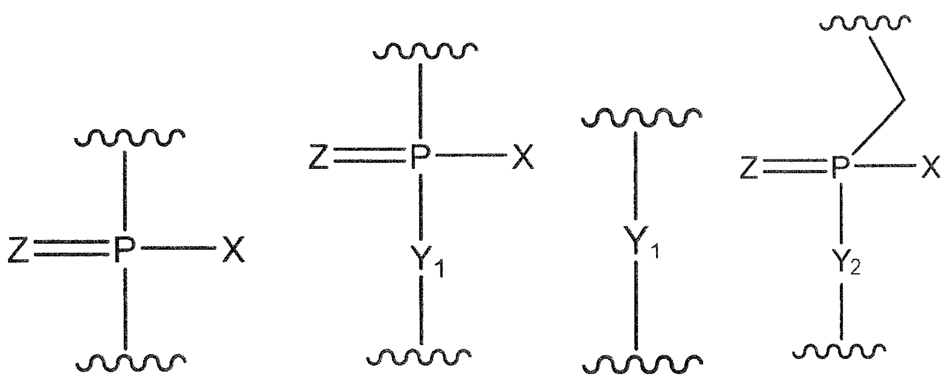
wherein Base represents a nucleobase.

[0055] The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (*e.g.*, AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (*e.g.*, Promega Inc., or Takara Co.), etc.

[0056] The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:

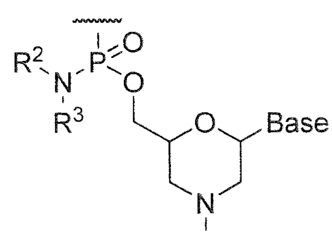


wherein Base has the same significance as defined above, and,
W represents a group shown by any one of the following groups:



wherein X represents $-CH_2R^1$, $-O-CH_2R^1$, $-S-CH_2R^1$, $-NR_2R^3$ or F;
R^1 represents H or an alkyl;
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;
Y_1 represents O, S, CH_2 or NR^1;
Y_2 represents O, S or NR^1;
Z represents O or S.

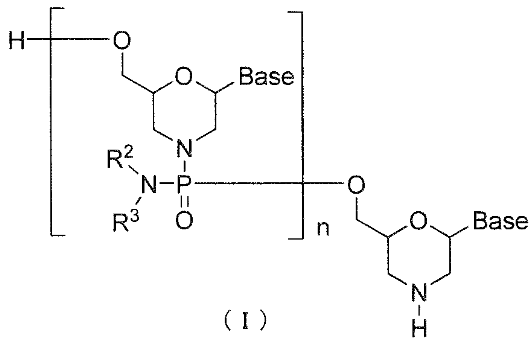
[0057] Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).



wherein Base, R^2 and R^3 have the same significance as defined above.
[0058] The morpholino oligomer may be produced in accordance with, *e.g.*, WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for producing PMO]

[0059] An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein Base, R^2 and R^3 have the same significance as defined above; and,
n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

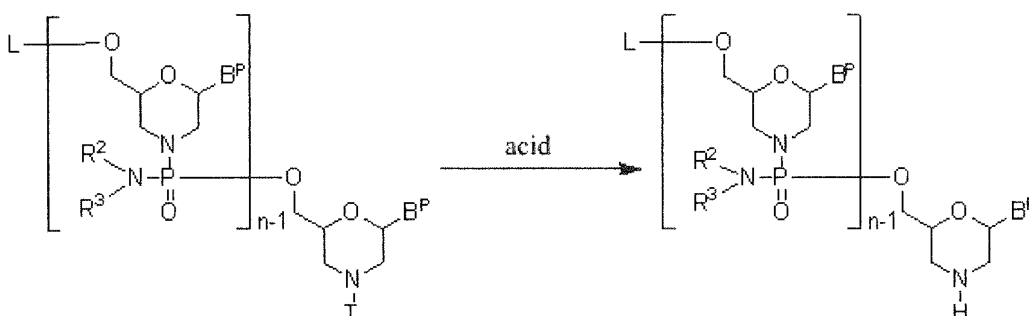
[0060] PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

[0061] The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

[0062] Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

[0063] The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II) (III)

wherein n, R^2 and R^3 have the same significance as defined above;
each B^P independently represents a nucleobase which may optionally be protected;
T represents trityl, monomethoxytrityl or dimethoxytrityl; and,
L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).

(IV)

[0064] The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

[0065] Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., *e.g.,* WO 2009/064471).

[0066] The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: *e.g.*, (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (*e.g.*, dichloromethane, acetonitrile, tetrazole, *N*-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (*e.g.*, aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin·HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (*e.g.*, Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (*e.g.*, NH_2-PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, *e.g.,* CPG), oxalyl-controlled pore glass (cf., *e.g.*, Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (*e.g.*, Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

[0067] A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

[0068] This step can be performed by reacting Compound (II) with an acid.

[0069] The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

[0070] An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, *e.g.,* 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

[0071] When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

[0072] The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

[0073] The reaction temperature in the reaction described above is preferably in a range of, *e.g.,* 10°C to 50°C, more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C.

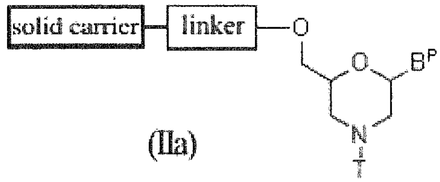
[0074] The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

[0075] After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

[0076] The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, *e.g.,* 10°C to 50°C, more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C.

[0077] The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

[0078] In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

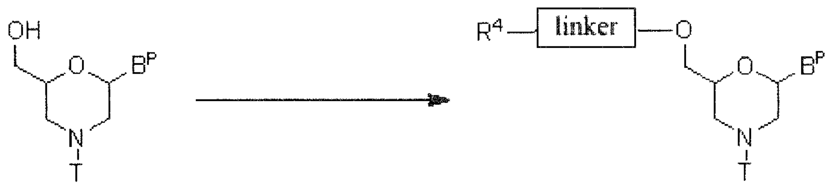


(IIa)

wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

[0079] The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).

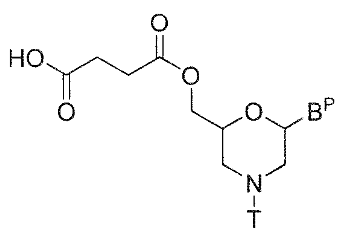


(V) (VI)

wherein B^P, T and linker have the same significance as defined above; and,
R^4 represents hydroxy, a halogen or amino.

[0080] This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

[0081] In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

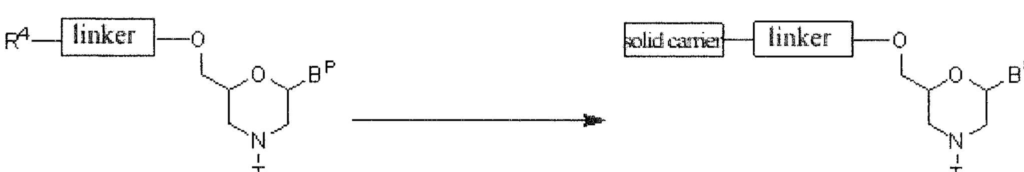


(VIa)

wherein B^P and T have the same significance as defined above.

Step 2:

[0082] Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).

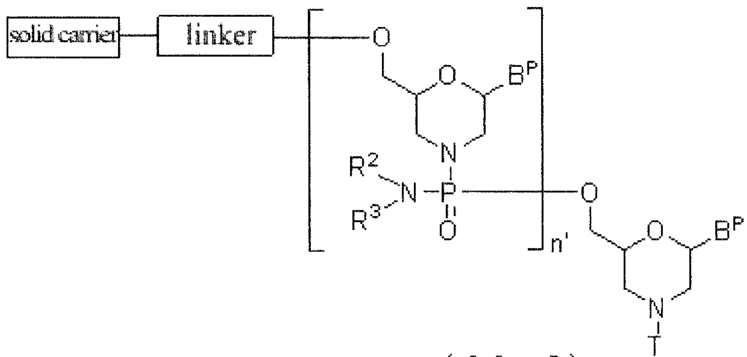


(VI) (IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

[0083] This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

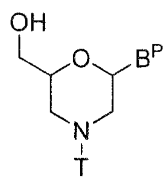
[0084] In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



(IIa2)

wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and, n' represents 1 to 98.

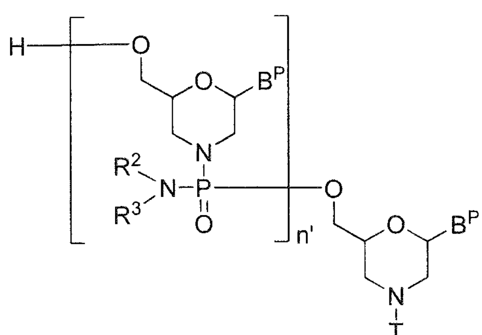
[0085] In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, *e.g.*, WO 1991/009033.



(IIb)

wherein B^P and T have the same significance as defined above.

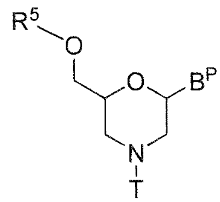
[0086] In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



(IIb2)

wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

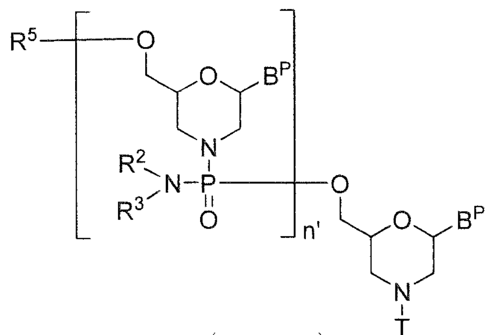
[0087] In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).



(I I c)

wherein B^P and T have the same significance as defined above; and,
R^5 represents an acyl.

[0088] In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

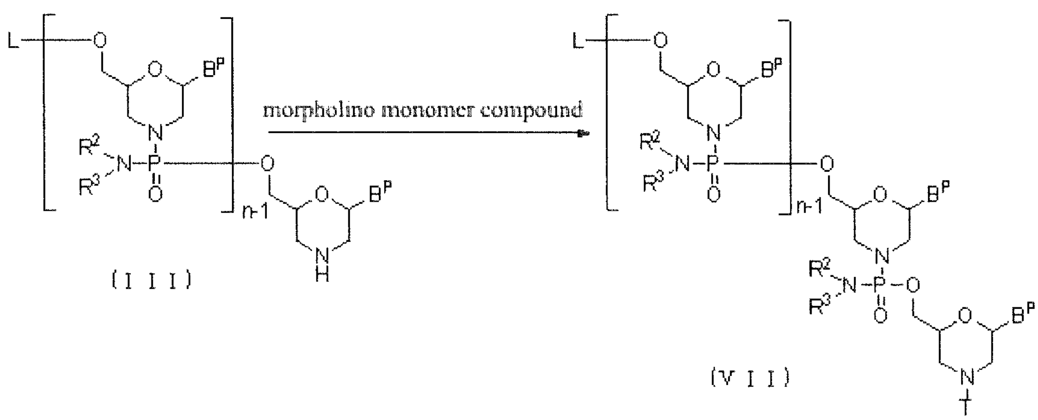


(I I c 2)

wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

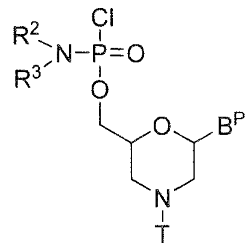
[0089] Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

[0090] This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

[0091] The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:



(VIII)

wherein B^P, R^2, R^3 and T have the same significance as defined above.

[0092] The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and *N*-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

[0093] The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, *N*-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

[0094] The reaction temperature is preferably in a range of, *e.g.,* 0°C to 100°C, and more preferably, in a range of 10°C to 50°C.

[0095] The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

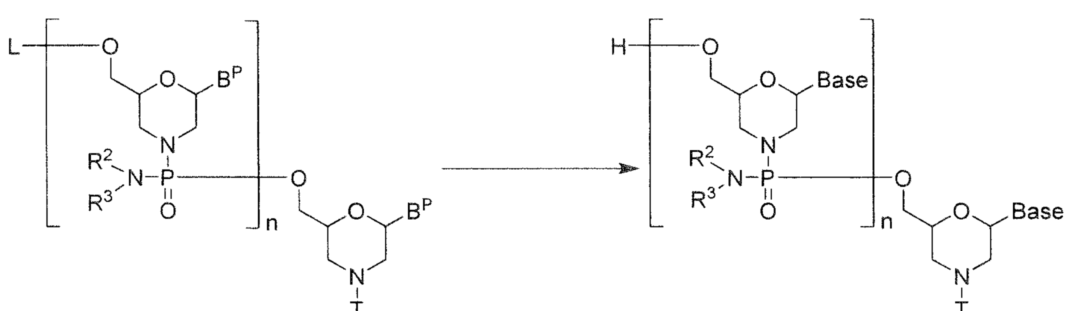
[0096] Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

[0097] If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, *N*-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, *e.g.,* 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

[0098] The reaction temperature in this reaction is preferably in a range of 10°C to 50°C, more preferably, in a range of 10°C to 50°C, much more preferably, in a range of 20°C to 40°C, and most preferably, in a range of 25°C to 35°C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

[0099] In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII) (IX)

wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

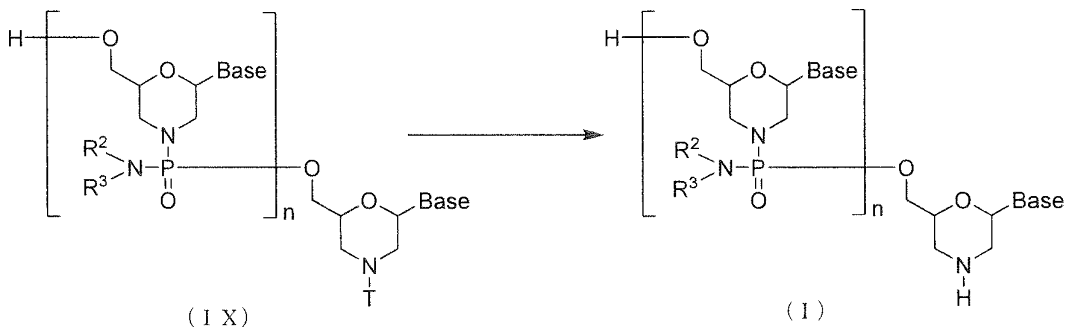
[0100] This step can be performed by reacting Compound (VII) with a deprotecting agent.

[0101] The "deprotecting agent" includes, *e.g.*, conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, *e.g.*, water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, *N*-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, *e.g.,* 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

[0102] The reaction temperature is appropriately in a range of 15°C to 75°C, preferably, in a range of 40°C to 70°C, and more preferably, in a range of 50°C to 60°C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

[0103] PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



wherein Base, n, R^2, R^3 and T have the same significance as defined above.

[0104] This step can be performed by adding an acid to Compound (IX).

[0105] The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

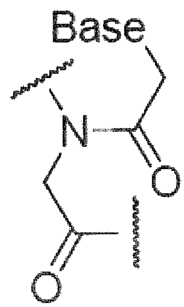
[0106] The reaction temperature is appropriately in a range of 10°C to 50°C, preferably, in a range of 20°C to 40°C, and more preferably, in a range of 25°C to 35°C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

[0107] PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., *e.g.*, WO 1991/09033).

[0108] In purification of PMO (I) using reversed phase chromatography, *e.g.*, a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

[0109] In purification of PMO (I) using ion exchange chromatography, *e.g.*, a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

[0110] A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:

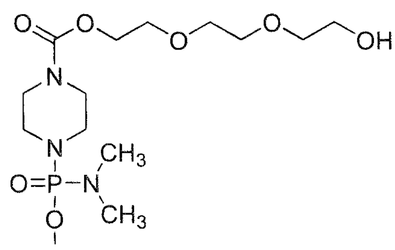


wherein Base has the same significance as defined above.

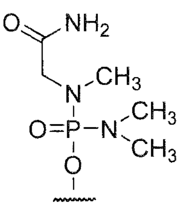
[0111] Peptide nucleic acids can be prepared by referring to, *e.g.,* the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J.Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

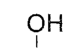
[0112] In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.



(1)



(2)



(3)

[0113] Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical composition

[0114] The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

[0115] In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

[0116] Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, *N*-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, *N, N'*-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, *N*-benzylphenethylamine, piperazine,

tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

[0117] Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

[0118] In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

[0119] A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

[0120] In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (*e.g.*, hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt% or less, preferably 70 wt% or less and more preferably, 50 wt% or less.

[0121] The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

[0122] The composition of the present invention may be prepared into, *e.g.,* a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about -40 to -20°C, performing a primary drying at 0 to 10°C under reduced pressure, and then performing a secondary drying at about 15 to 25°C under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

[0123] The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution

by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water , physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

[0124] It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

[0125] In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

[0126] Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

[EXAMPLES]

[REFERENCE EXAMPLE 1]

<u>4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy} -4-oxobutanoic acid loaded onto aminomethyl polystyrene resin</u>

Step 1: Production of

4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-l(2H)-yl)-4-tritylmorpholin-2-yl]meth oxy}-4-oxobutanoic acid

[0127] Under argon atmosphere, 22.0 g of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimid in-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of

4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy} -4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

[0128] After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]meth oxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A 1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin

was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

[0129] The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV measurement

[0130]

Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

[REFERENCE EXAMPLE 2]

4-Oxo-4-1[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpho lin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

[0131] Guanosine, 100 g, was dried at 80°C under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0°C, followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf. :Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2:

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

[0132] In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4°C overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).
^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J = 7.82Hz), 7.00 (3H, m), 5.95 (1H, dd, J = 10.64, 2.42Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

[0133] In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature

for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of

4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

[0134] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that *N*-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[REFERENCE EXAMPLE 3]

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

[0135] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4 (1H,3H)-dione was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[REFERENCE EXAMPLE 4]

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

[0136] The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

[0137] According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31 - 55	5' end: group (3)	SEQ ID NO: 4
2	32 - 53	5' end: group (3)	SEQ ID NO: 8
3	32 - 56	5' end: group (3)	SEQ ID NO: 11
4	33 - 54	5' end: group (3)	SEQ ID NO: 15
5	34 - 58	5' end: group (3)	SEQ ID NO: 25
6	36 - 53	5' end: group (3)	SEQ ID NO: 32

(continued)

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
7	36 - 55	5' end: group (3)	SEQ ID NO: 34
8	36 - 56	5' end: group (3)	SEQ ID NO: 35
9	36 - 57	5' end: group (3)	SEQ ID NO: 36
10	33 - 57	5' end: group (3)	SEQ ID NO: 18
11	39 - 69	Sequence corresponding to H53A(+39+69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30 - 59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32 - 56	5' end: group (1)	SEQ ID NO: 11
14	36 - 56	5' end: group (1)	SEQ ID NO: 35
15	30 - 59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23 - 47	Sequence corresponding to SEQ ID NO:429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

[EXAMPLE 1]

PMO No. 8

[0138] 4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2 -yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5

(continued)

Step	Reagent	Volume (mL)	Time (min)
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6 - 11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

[0139] The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2, 6-lutidine in dichloromethane.

[0140] The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55°C for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid - triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, φ50x 100 mm, 1CV=200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50% /9CV

[0141] Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M

sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

[0142] The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, φ40x 150 mm, 1CV=200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35% /15CV

[0143] Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1 M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

[0144] The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82

Found: 6923.54

[EXAMPLE 2]

PMO. No. 1

[0145] The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96

Found: 8296.24

[EXAMPLE 3]

PMO. No. 2

[0146] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13

Found: 7309.23

[EXAMPLE 4]

PMO. No. 3

[0147] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94
Found: 8270.55

[EXAMPLE 5]

PMO. No. 4

[0148] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholi n-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13
Found: 7310.17

[EXAMPLE 6]

PMO. No. 5

[0149] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholi n-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94
Found: 8270.20

[EXAMPLE 7]

PMO. No. 6

[0150] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01
Found: 5963.68

[EXAMPLE 8]

PMO. No. 7

[0151] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55
Found: 6608.85

[EXAMPLE 9]

PMO. No. 9

[0152] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11

Found: 7279.42

[EXAMPLE 10]

PMO. No. 10

[0153] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9 (6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95
Found: 8295.91

[EXAMPLE 11]

PMO. No. 13

[0154] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15

Found: 7276.69

[EXAMPLE 12]

PMO. No. 14

[0155] The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFEENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27

Found: 8622.29

[COMPARATIVE EXAMPLE 1]

PMO. No. 11

[0156] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63

Found: 10273.71

[COMPARATIVE EXAMPLE 2]

PMO.No.15

[0157] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33

Found: 9940.77

[COMPARATIVE EXAMPLE 3]

PMO.No.16

[0158] The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94
Found: 8238.69

[TEST EXAMPLE 1]

In vitro assay

[0159] Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4x 10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

[0160] After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0161] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
94°C, 2 mins: thermal denaturation
[94°C, 10 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0162] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

[0163] Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94°C, 2 mins: thermal denaturation
[94°C, 15 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0164] The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0165] The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0166] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0167] The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

[TEST EXAMPLE 2]

In vitro assay using human fibroblasts

[0168] Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

[0169] After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5x $10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

[0170] The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

[0171] Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 1 mins; 60°C, 1 mins; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 7 mins: final extension

[0172] The primers used were hEX51F and hEX55R.

hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3' (SEQ ID NO: 45)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

[0173] The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0174] The results are shown in FIGS. 2 and 3. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. 2) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 2). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 2).

[0175] Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. 3) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 3). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 3).

[TEST EXAMPLE 3]

In vitro assay using human fibroblasts

[0176] The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen 1 coexpression retroviral vector.

[0177] After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5x $10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

[0178] The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

[0179] Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 1 mins; 60°C, 1 mins; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 7 mins: final extension

[0180] The primers used were hEx44F and h55R.

hEx44F: 5'- TGTTGAGAAATGGCGGCGT-3' (SEQ ID NO: 48)
hEx55R: 5'- TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

[0181] The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0182] The results are shown in FIGS. 4 and 5. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. 4) or deletion of exons 48-52 (FIG. 5). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. 4).

[TEST EXAMPLE 4]

Western blotting

[0183] The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by

Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

[0184] The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental results

[0185] The results are shown in FIGS. 6 and 7. In this experiment it was confirmed by western blotting (FIG. 6) and immunostaining (FIG. 7) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

[TEST EXAMPLE 5]

In vitro assay using human fibroblasts

[0186] The experiment was performed as in TEST EXAMPLE 3.

Experimental results

[0187] The results are shown in FIG. 8. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. 8).

[TEST EXAMPLE 6]

In vitro assay

[0188] Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58

(continued)

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96

(continued)

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

[0189] RD cells (human rhabdomyosarcoma cell line) were plated at 3x 10^5 in a 6-well plate and cultured in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37°C and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

[0190] After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0191] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50°C, 30 mins: reverse transcription
94°C, 2 mins: thermal denaturation
[94°C, 10 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0192] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0193] Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94°C, 2 mins: thermal denaturation
[94°C, 15 seconds; 58°C, 30 seconds; 68 °C, 45 seconds] x 30 cycles: PCR amplification
68°C, 7 mins: final extension

[0194] The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

[0195] The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0196] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Experimental results

[0197] The results are shown in FIGS. 9 to 17. These experiments revealed that, when the antisense oligomers were designed at positions 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping is caused with a high efficiency.

[TEST EXAMPLE 7]

[0198] Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5x 10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

[0199] After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

[0200] One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows..

50°C, 30 mins: reverse transcription
95°C, 15 mins: thermal denaturation
[94°C, 30 seconds; 60°C, 30 seconds; 72 °C, 1 mins] x 35 cycles: PCR amplification
72°C, 10 mins: final extension

[0201] The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

[0202] The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

[0203] The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency } (\%) = A/(A + B) \times 100$$

Experimental results

[0204] The results are shown in FIGS. 18 and 19. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. 18). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. 19). These results showed that the sequences with -OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

[0205] Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

[0206] Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence listing free text

[0207]

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SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
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SEQUENCE LISTING

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Claims

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a

nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

2. The antisense oligomer according to claim 1, which is an oligonucleotide.

3. The antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

4. The antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Patentansprüche

1. Antisense-Oligomer, das das Überspringen des 53. Exons in dem humanen Dystrophin-Gen bewirkt, bestehend aus einer Nukleotidsequenz, die komplementär zu dem 36. bis 60. Nukleotid des 5'-Endes des 53. Exons in dem humanen Dystrophin-Gen ist.

2. Antisense-Oligomer nach Anspruch 1, wobei es sich um ein Oligonukleotid handelt.

3. Antisense-Oligomer nach Anspruch 2, wobei die Zuckereinheit und/oder die Phosphatbinderegion von mindestens einem Nukleotid, das das Oligonukleotid darstellt, modifiziert ist.

4. Antisense-Oligomer nach Anspruch 3, wobei die Zuckereinheit von mindestens einem Nukleotid, das das Oligonukleotid darstellt, eine Ribose ist, in der die 2'-OH-Gruppe durch ein beliebiges ersetzt ist, das aus der Gruppe ausgewählt ist, die aus Folgendem besteht: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br und I, wobei R ein Alkyl oder ein Aryl ist und R' ein Alkylen ist.

Revendications

1. Oligomère anti-sens qui provoque le saut du 53ème exon dans le gène humain de la dystrophine, constitué d'une séquence nucléotidique complémentaire du 36ème au 60ème nucléotides à partir de l'extrémité 5' du 53ème exon du gène humain de la dystrophine.

2. Oligomère anti-sens selon la revendication 1, qui est un oligonucléotide.

3. Oligomère anti-sens selon la revendication 2, dans lequel le groupement sucre et/ou la région de liaison au phosphate d'au moins un nucléotide constituant l'oligonucléotide est modifié.

4. Oligomère anti-sens selon la revendication 3, dans lequel le groupement sucre d'au moins un nucléotide constituant l'oligonucléotide est un ribose dans lequel le groupe 2'-OH est remplacé par l'un quelconque sélectionné parmi le groupe constitué de : OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, et I, dans lequel R est un alkyle ou un aryle et R' est un alkylène.

Figure 1

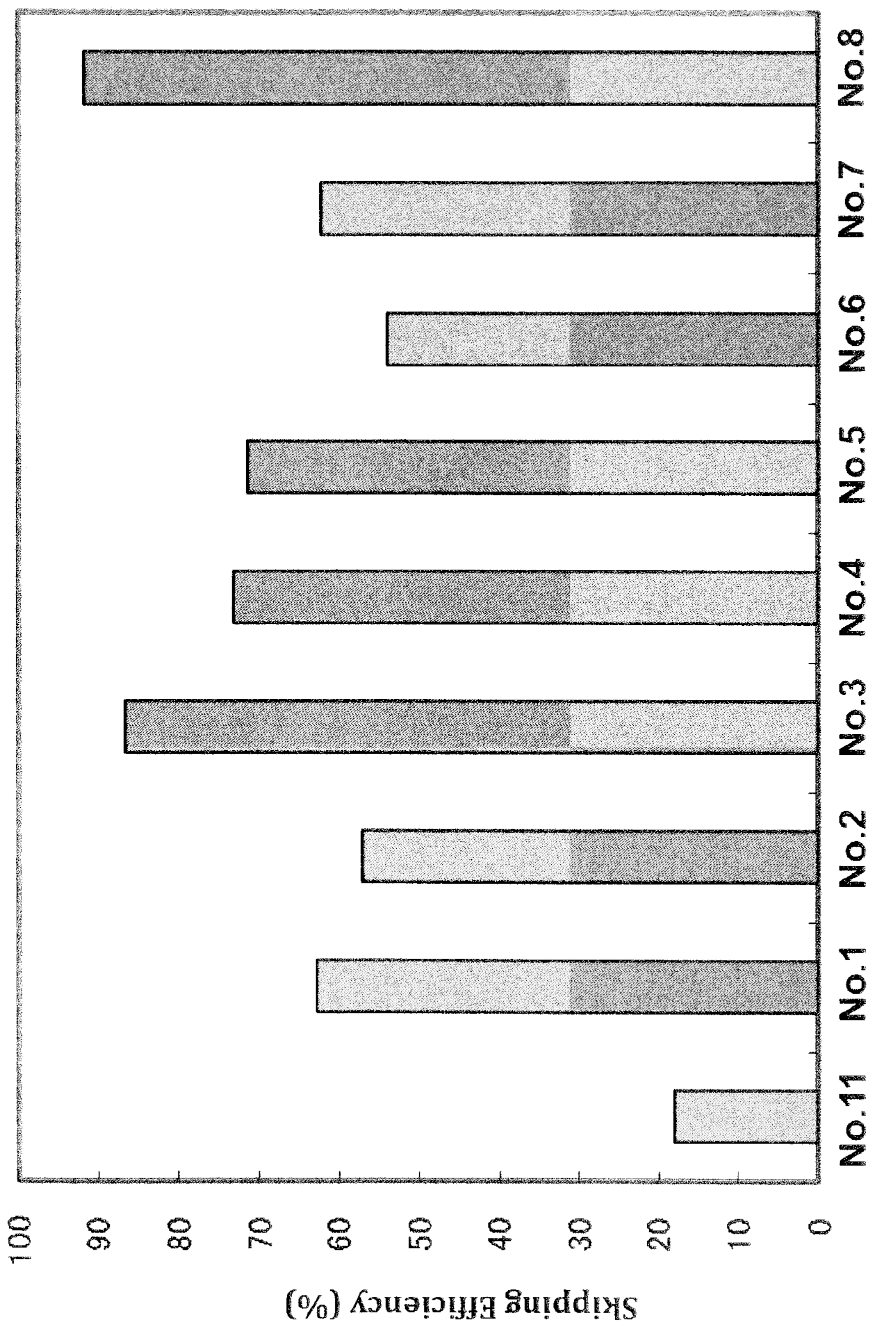

Skipping Efficiency (%)
0
10
20
30
40
50
60
70
80
90
100
No.11
No.1
No.2
No.3
No.4
No.5
No.6
No.7
No.8

Figure 2

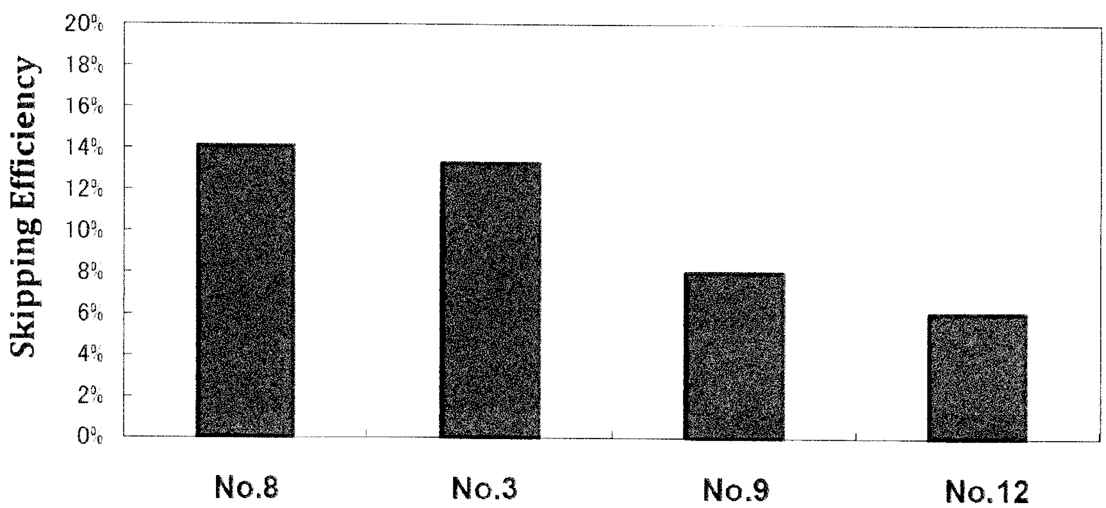


Figure 3

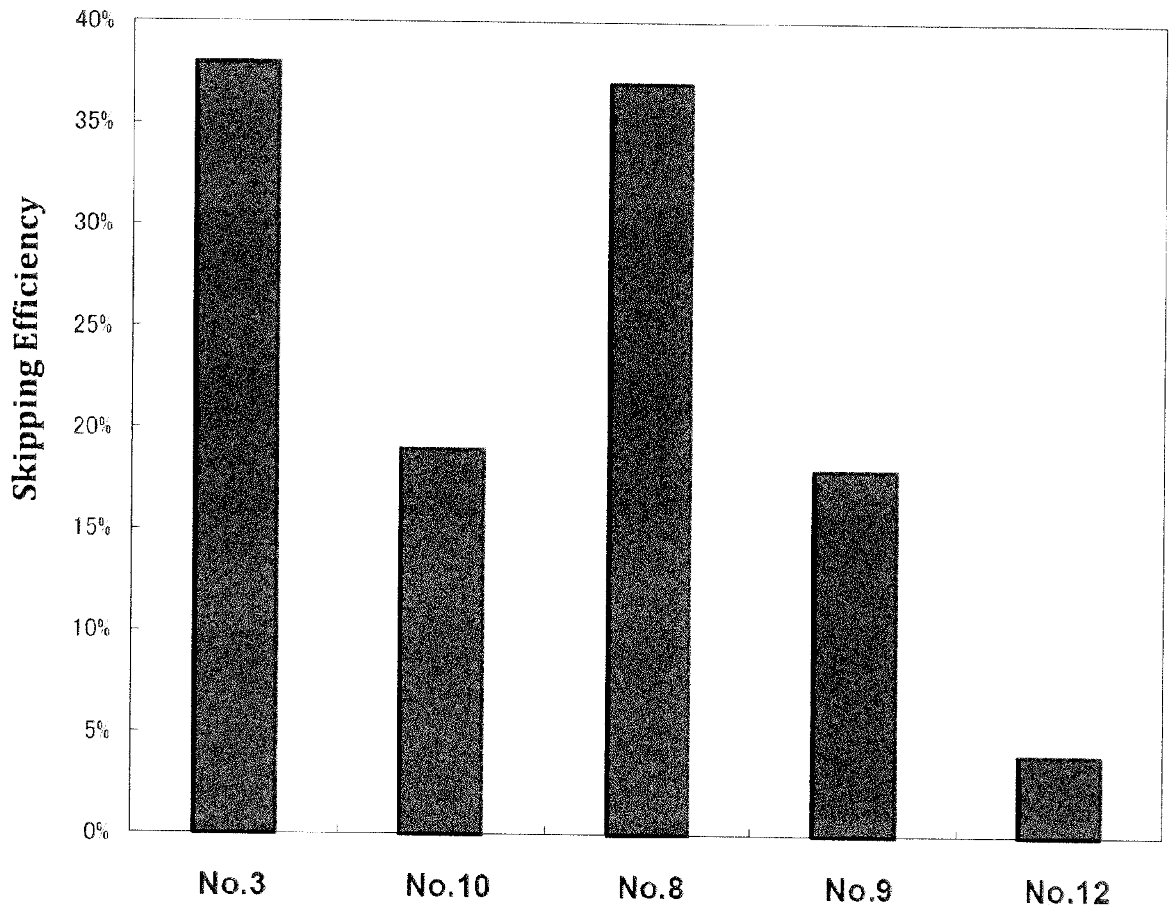

Skipping Efficiency
40%
35%
30%
25%
20%
15%
10%
5%
0%
No.3
No.10
No.8
No.9
No.12

Figure 4

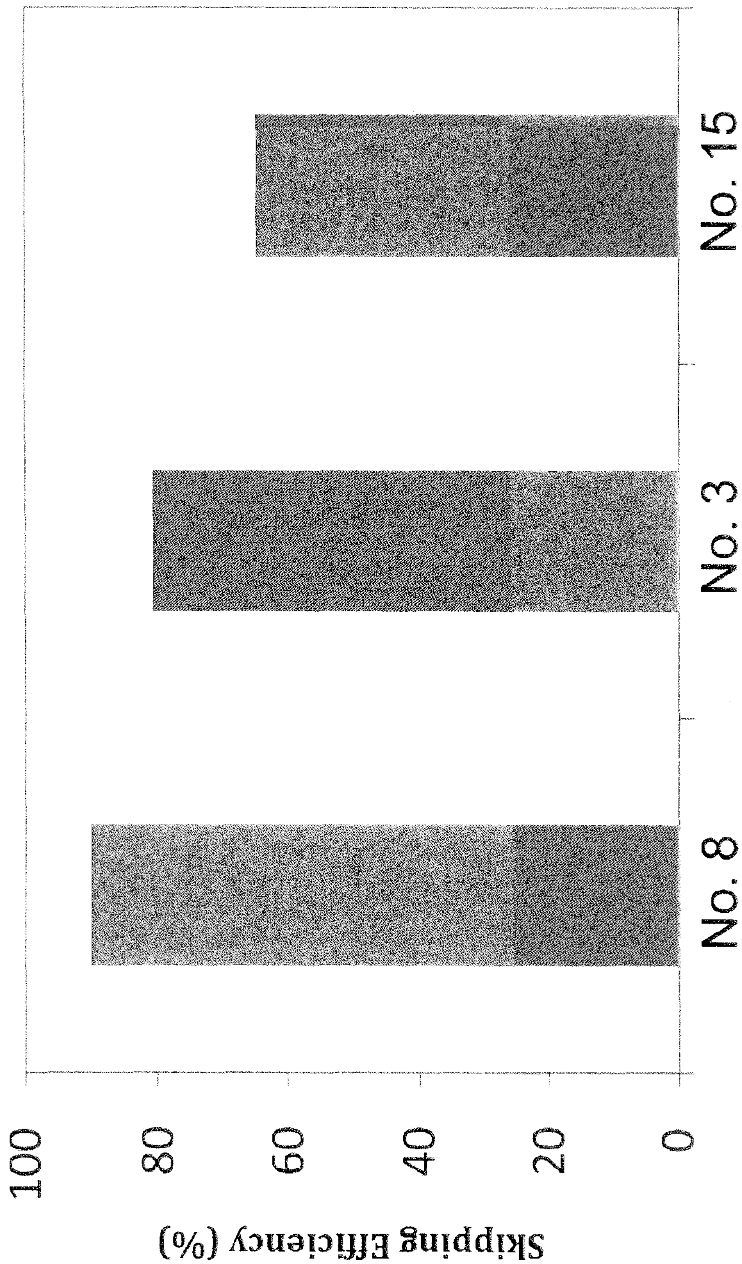


Figure 5

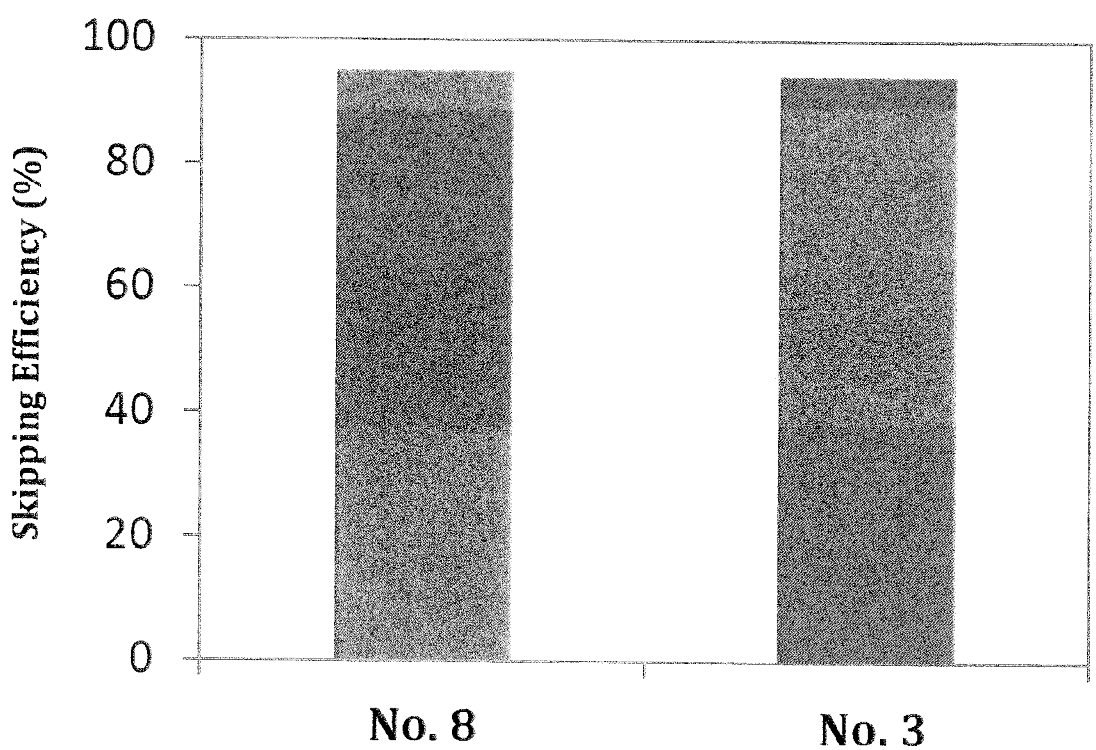


Figure 6

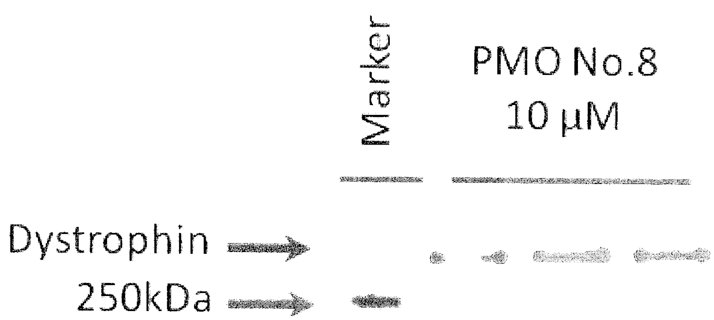


Figure 7

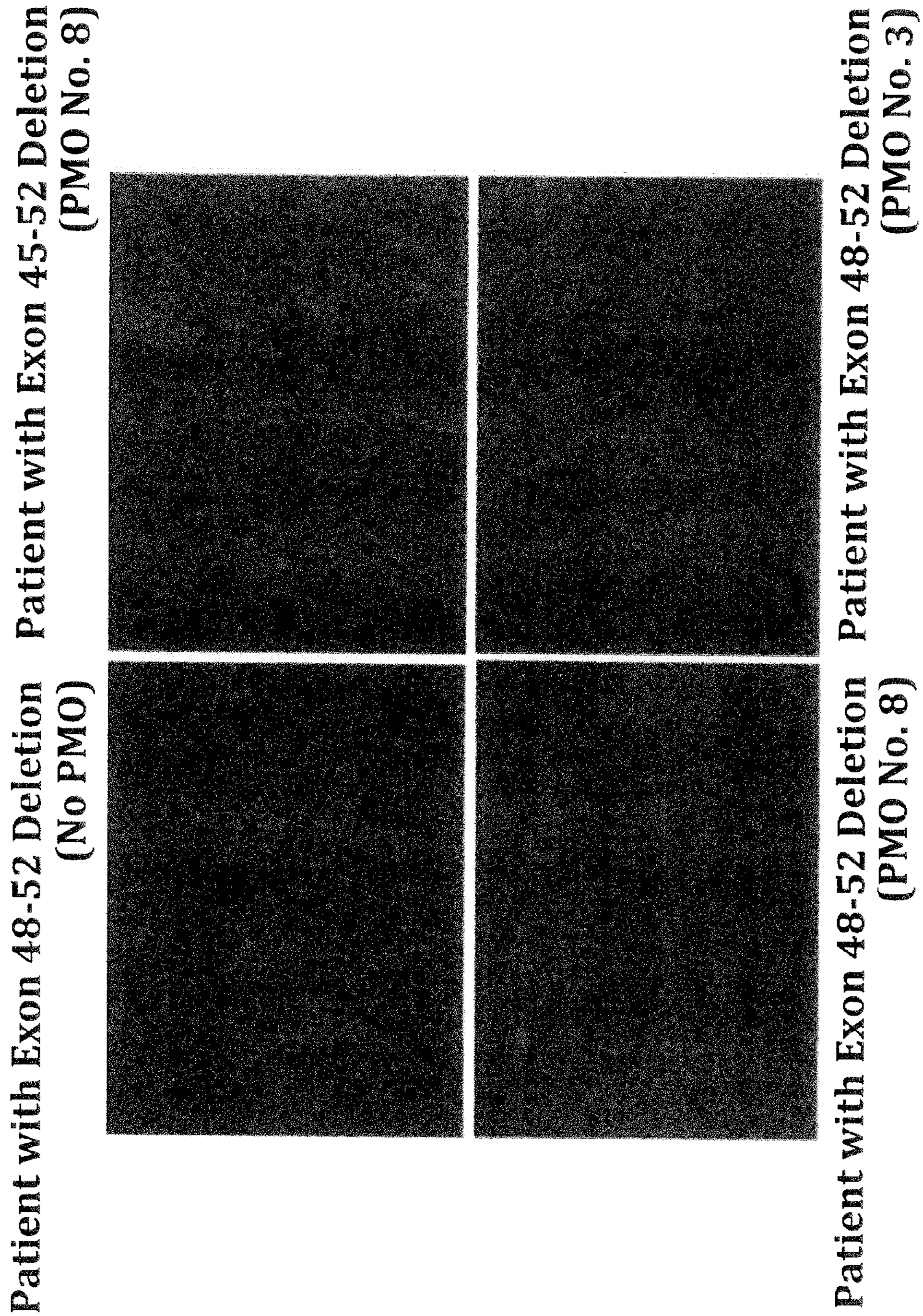

Patient with Exon 45-52 Deletion (PMO No. 8)
Patient with Exon 48-52 Deletion (PMO No. 3)
Patient with Exon 48-52 Deletion (No PMO)
Patient with Exon 48-52 Deletion (PMO No. 8)

Figure 8

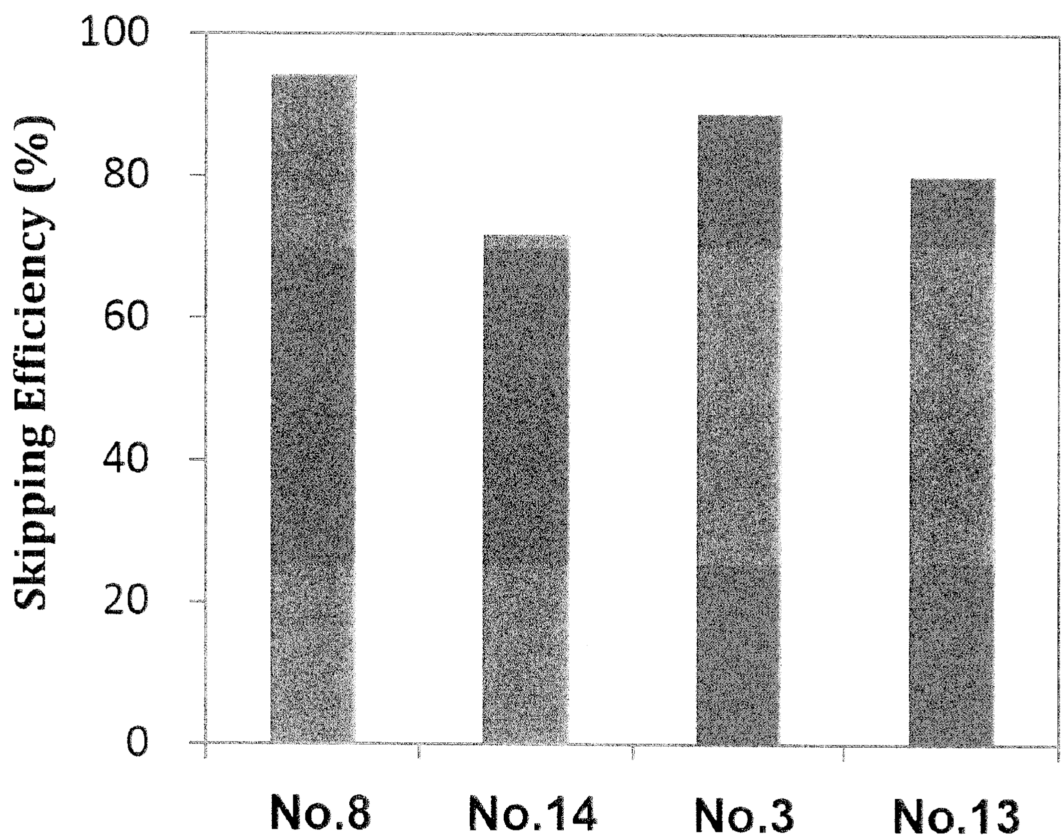


Figure 9

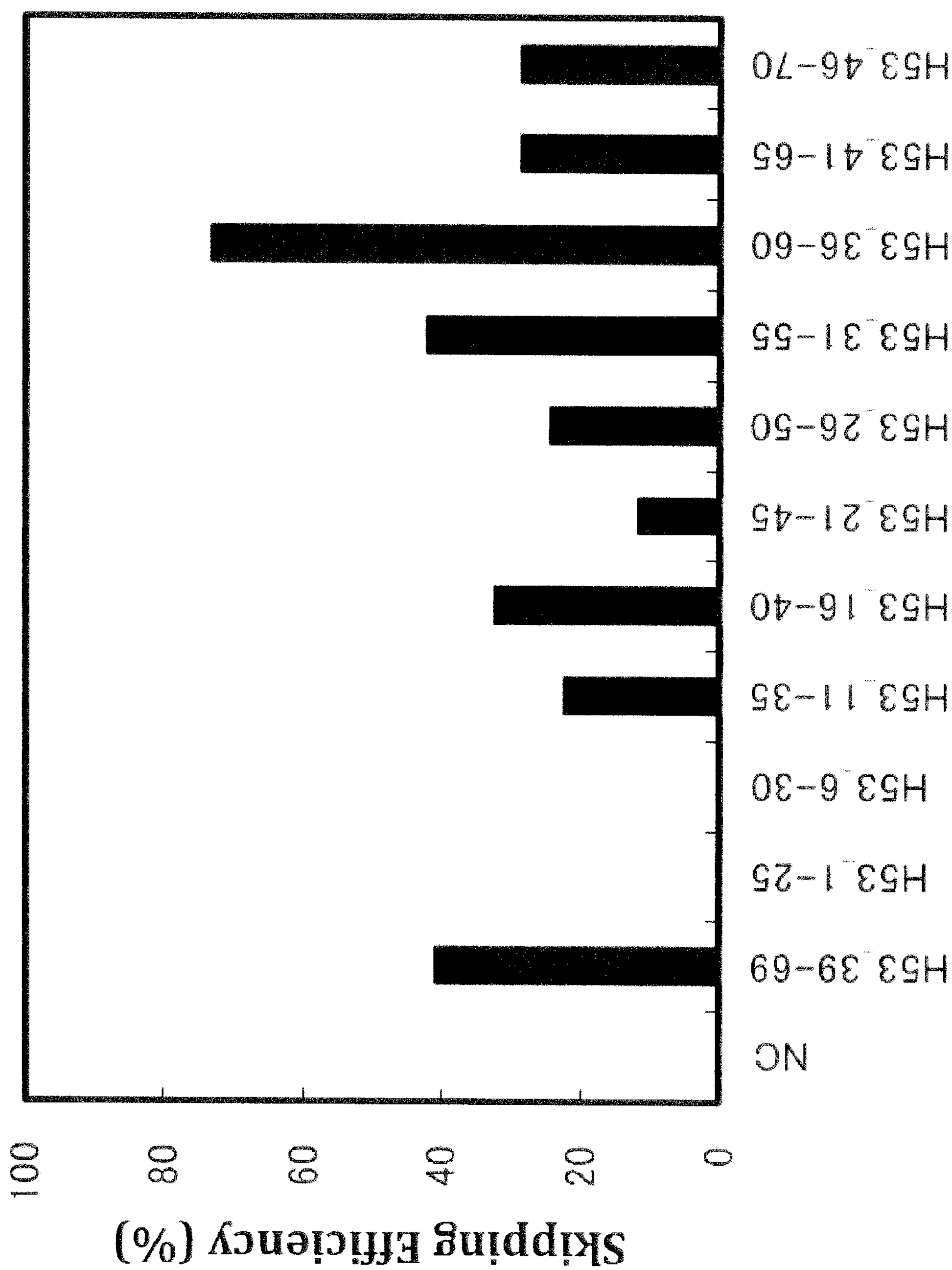

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_1-25
H53_6-30
H53_11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

Figure 10

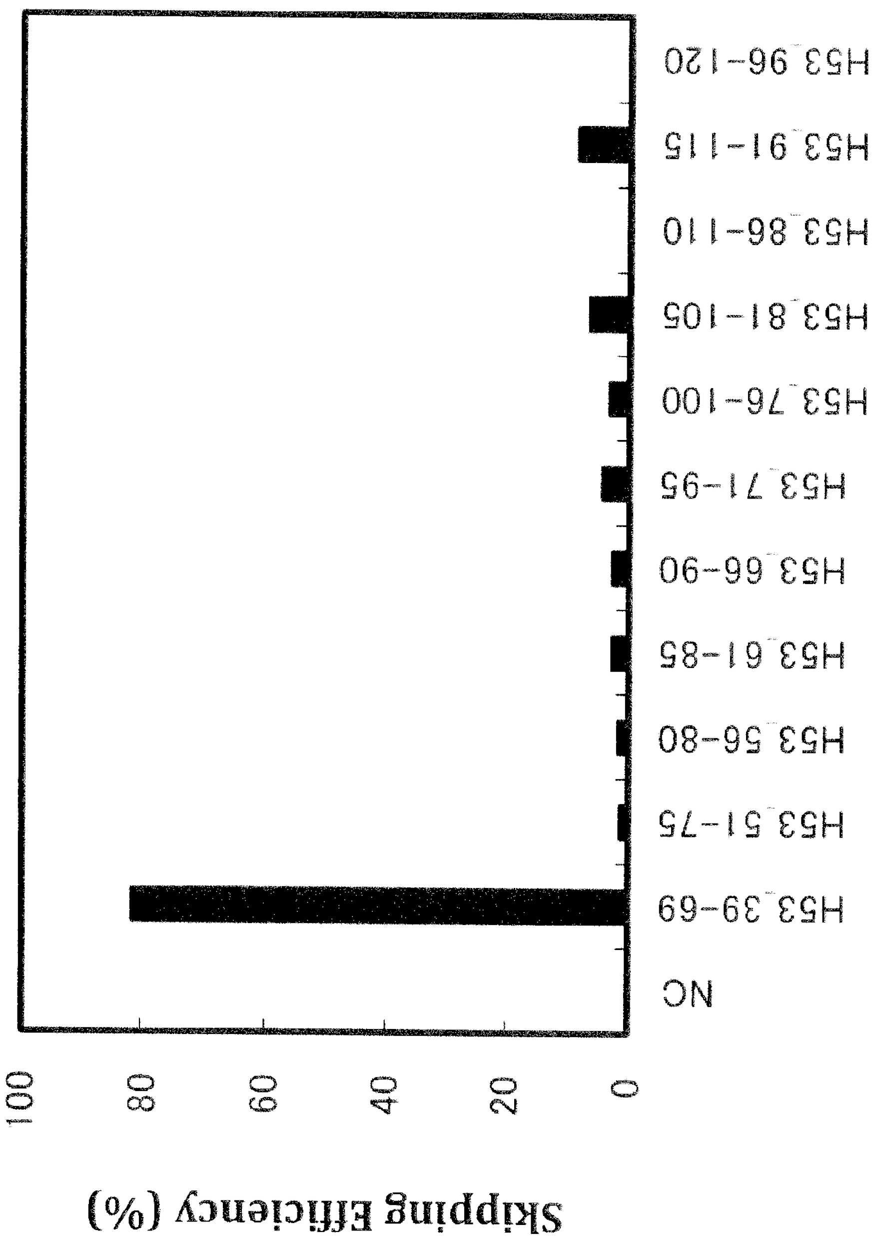

H53_96-120
H53_91-115
H53_86-110
H53_81-105
H53_76-100
H53_71-95
H53_66-90
H53_61-85
H53_56-80
H53_51-75
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 11

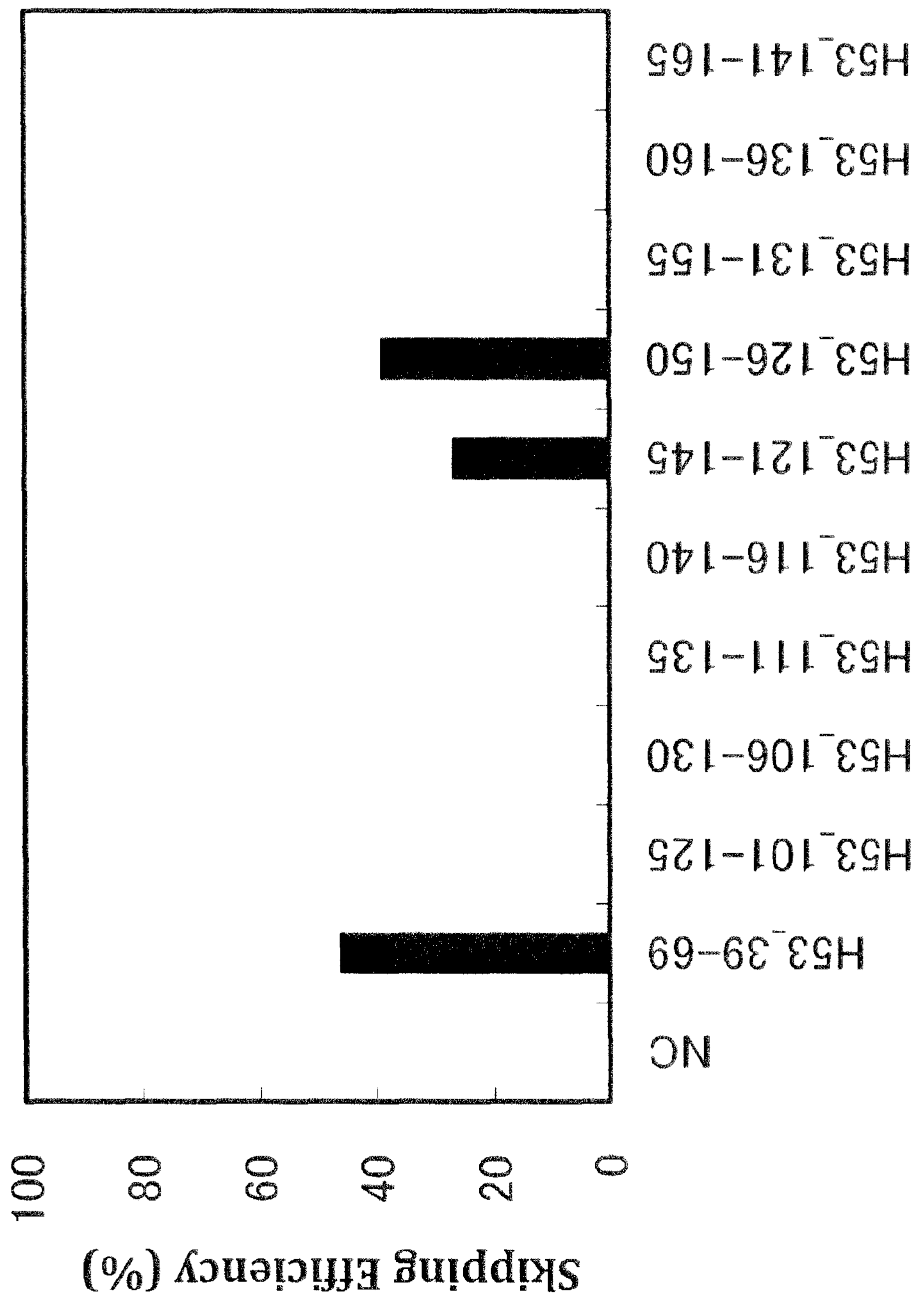

H53_141-165
H53_136-160
H53_131-155
H53_126-150
H53_121-145
H53_116-140
H53_111-135
H53_106-130
H53_101-125
H53_39-69
NC
0
20
40
60
80
100
Skipping Efficiency (%)

Figure 12

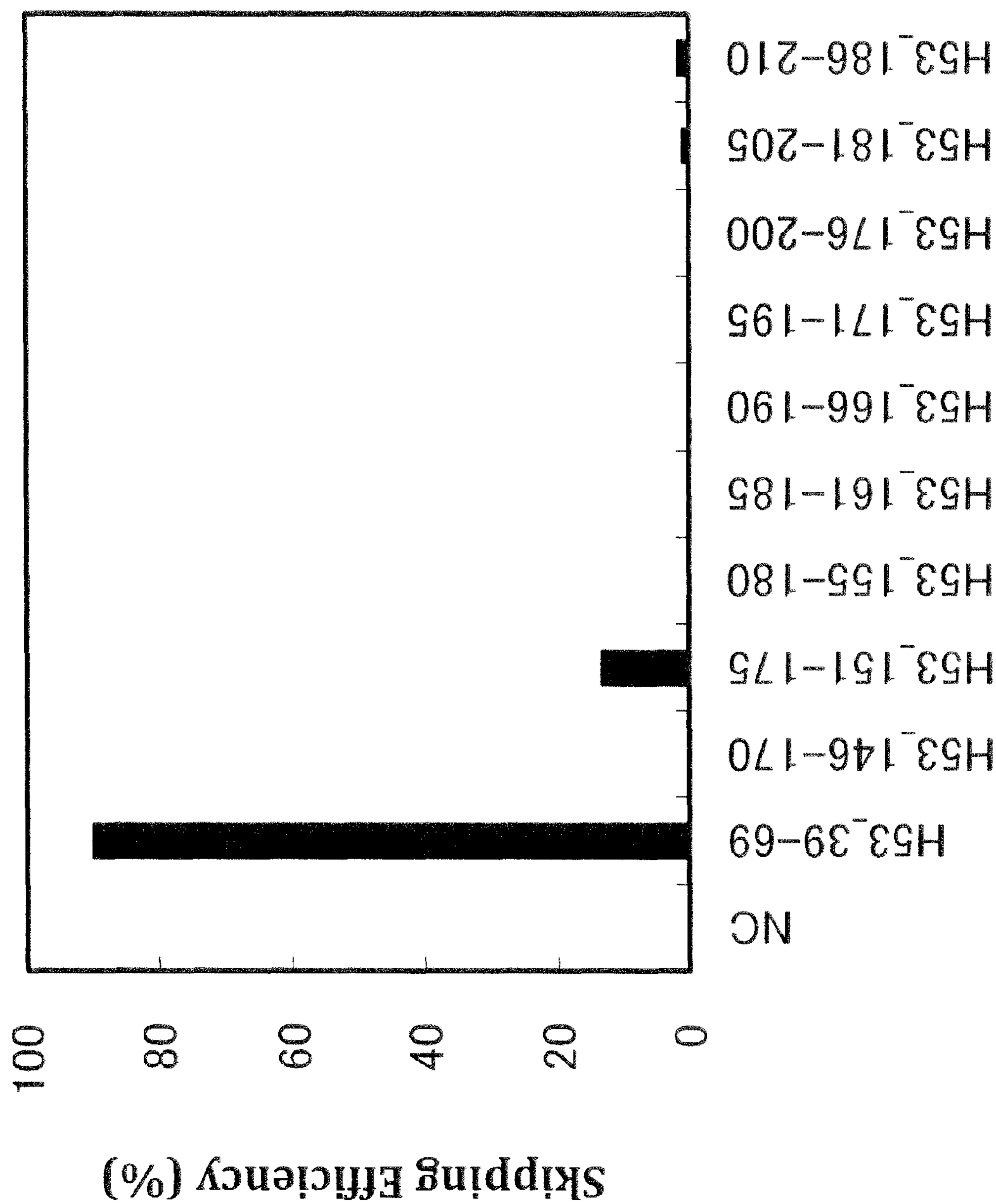

H53_186-210
H53_181-205
H53_176-200
H53_171-195
H53_166-190
H53_161-185
H53_155-180
H53_151-175
H53_146-170
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 13

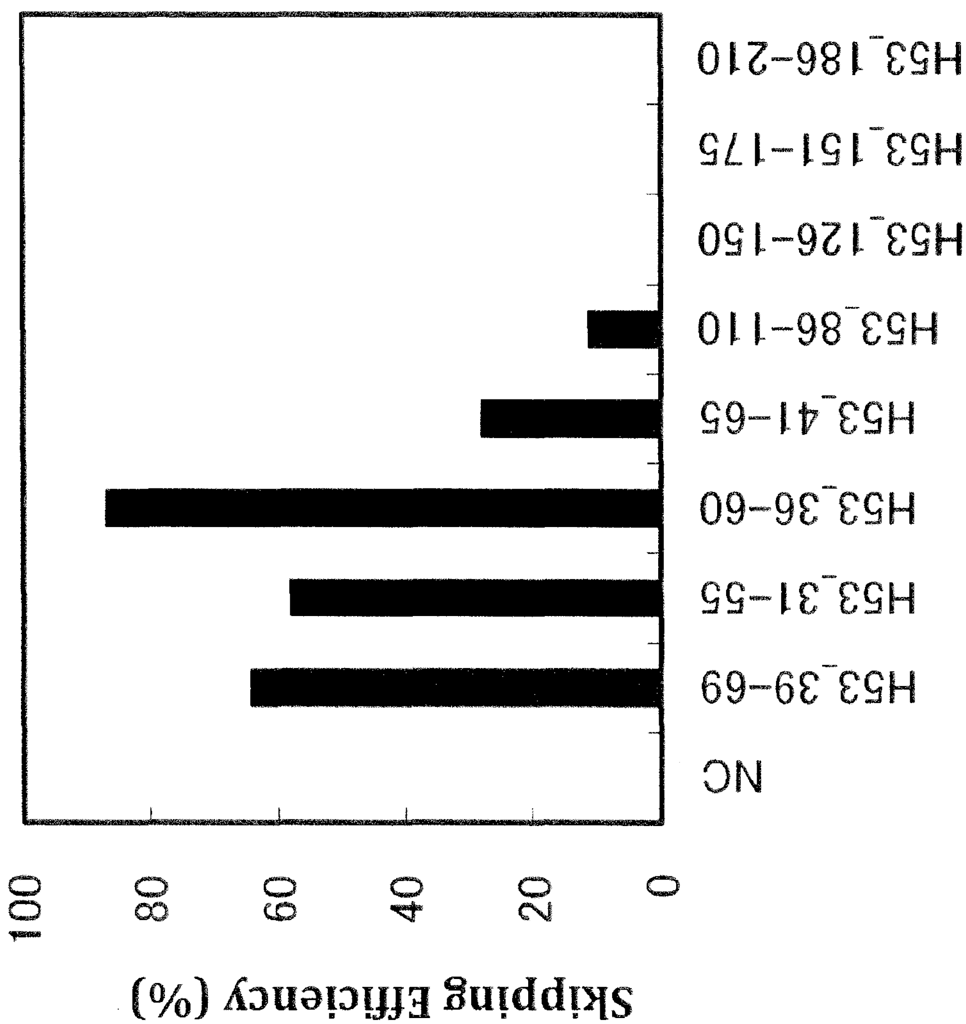

H53_186-210
H53_151-175
H53_126-150
H53_86-110
H53_41-65
H53_36-60
H53_31-55
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 14

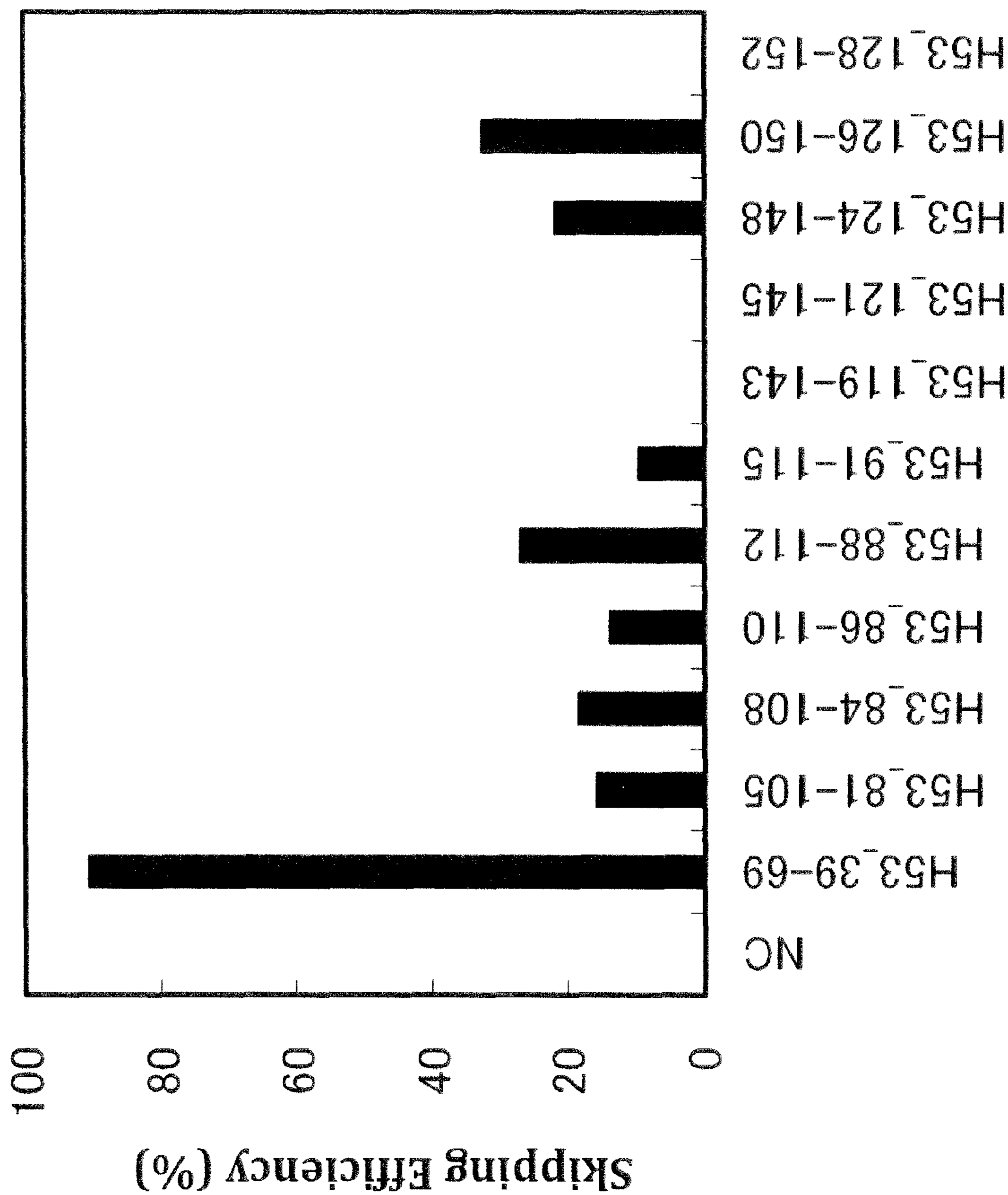

H53_128-152
H53_126-150
H53_124-148
H53_121-145
H53_119-143
H53_91-115
H53_88-112
H53_86-110
H53_84-108
H53_81-105
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 15

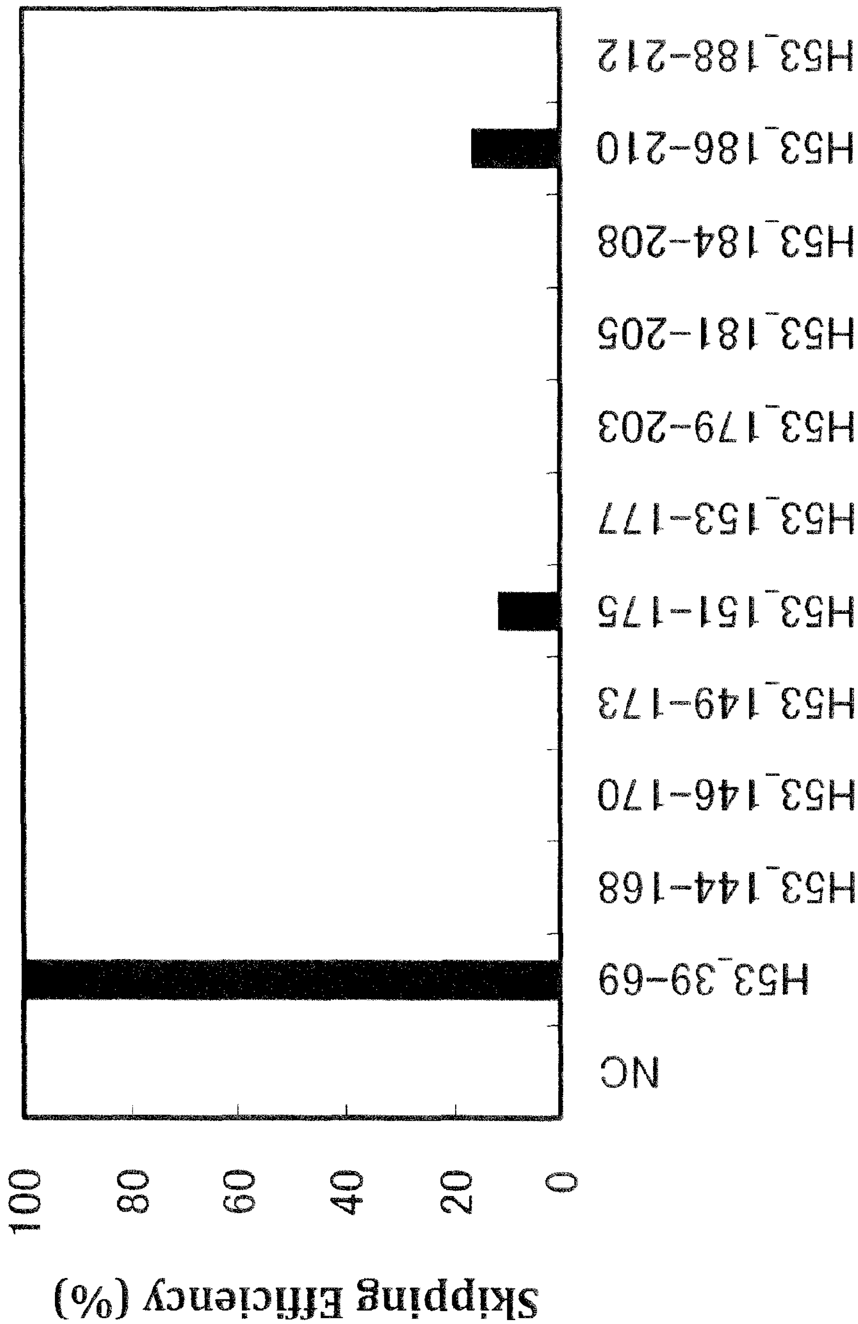

H53_188-212
H53_186-210
H53_184-208
H53_181-205
H53_179-203
H53_153-177
H53_151-175
H53_149-173
H53_146-170
H53_144-168
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 16

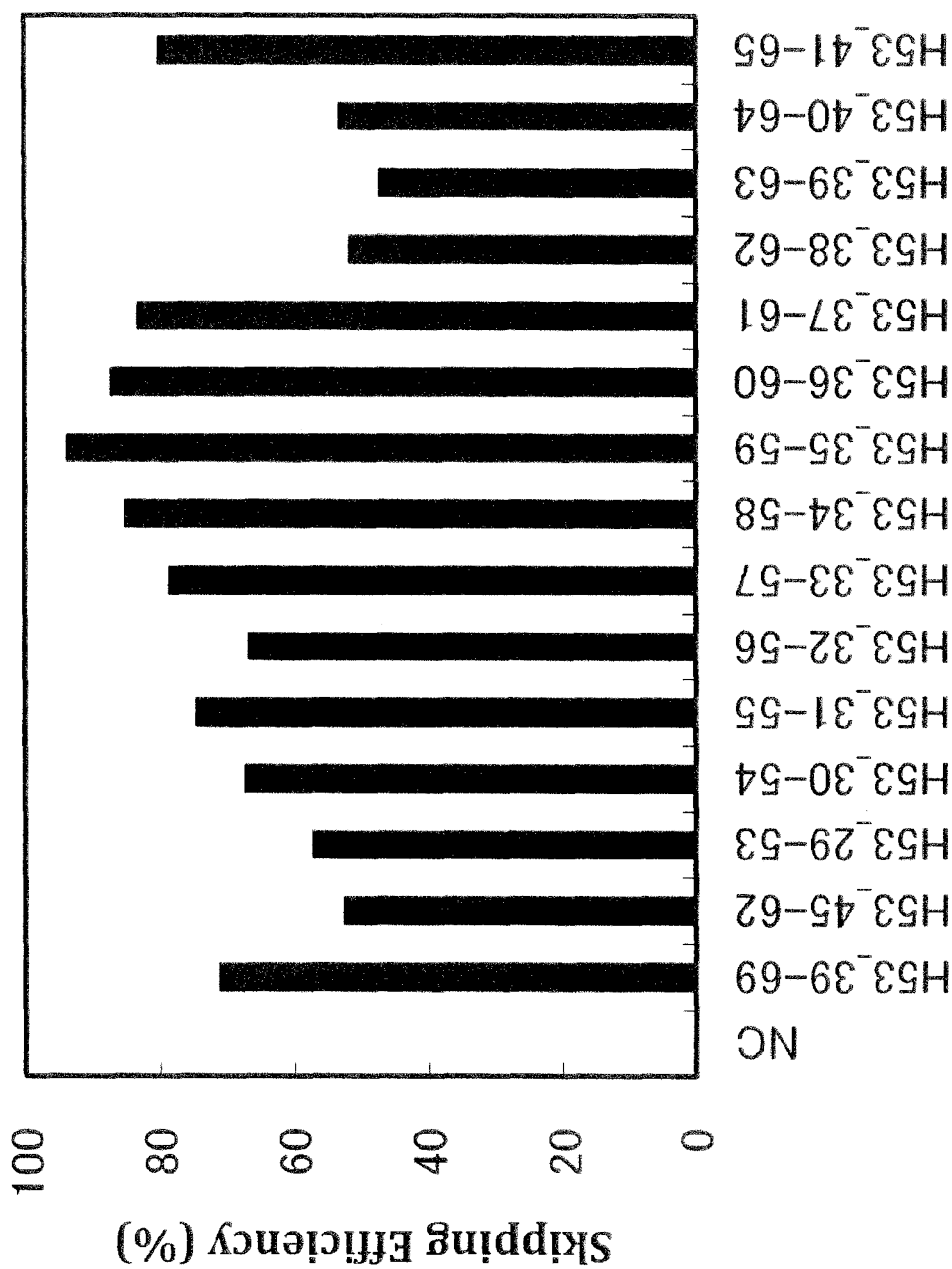

H53_41-65
H53_40-64
H53_39-63
H53_38-62
H53_37-61
H53_36-60
H53_35-59
H53_34-58
H53_33-57
H53_32-56
H53_31-55
H53_30-54
H53_29-53
H53_45-62
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 17

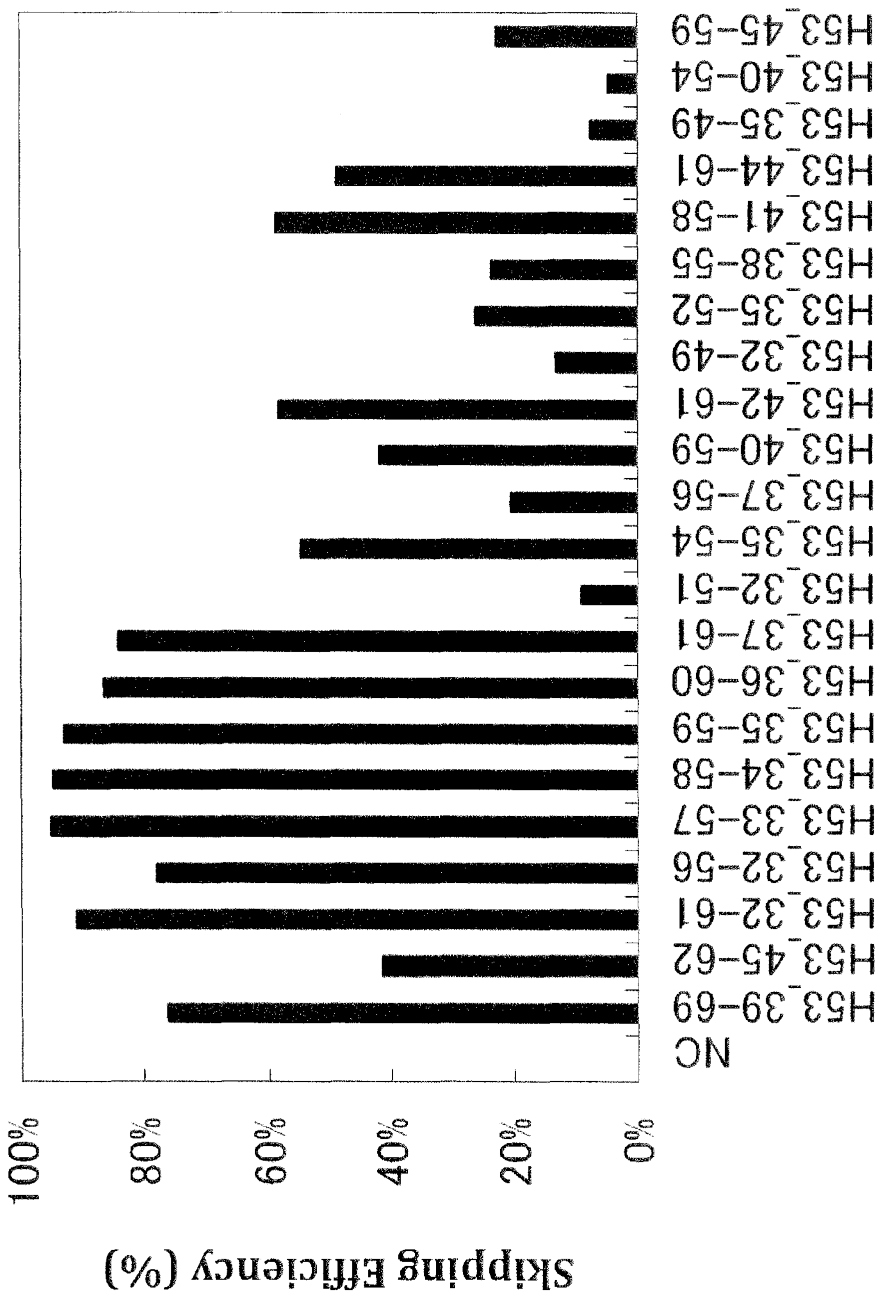

NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
H53_35-52
H53_38-55
H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59
0%
20%
40%
60%
80%
100%
Skipping Efficiency (%)

Figure 18

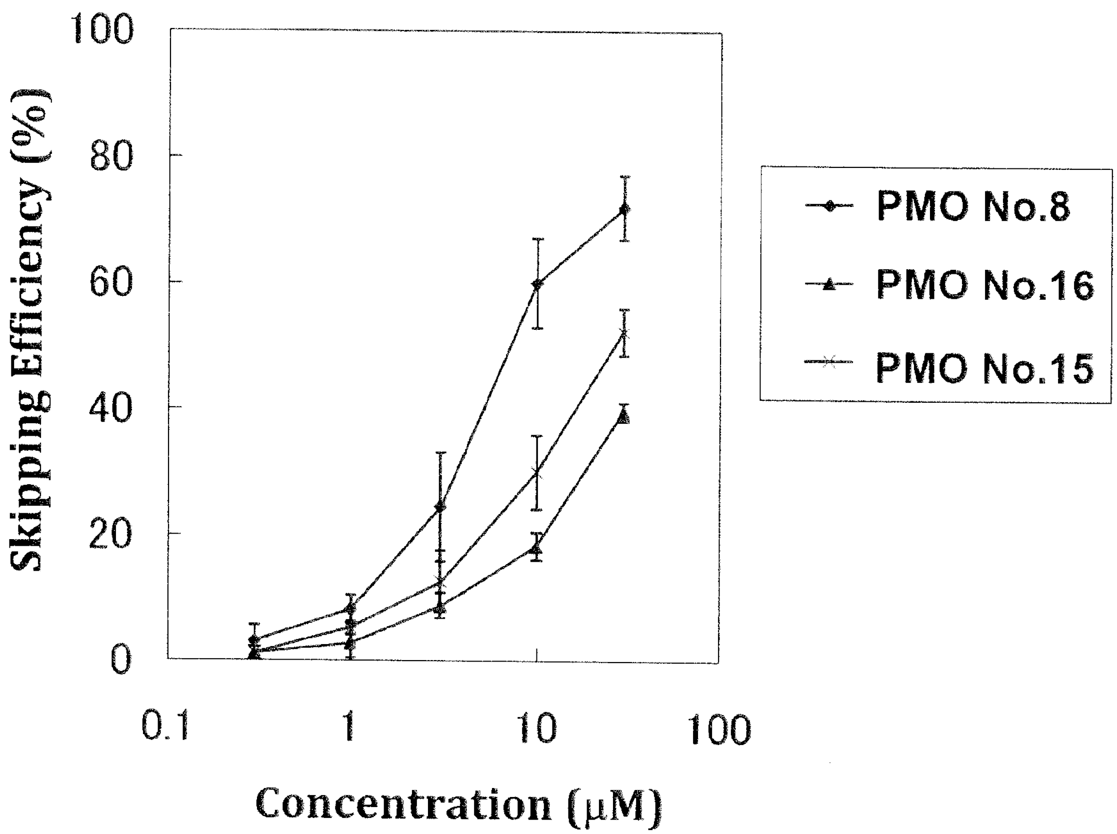
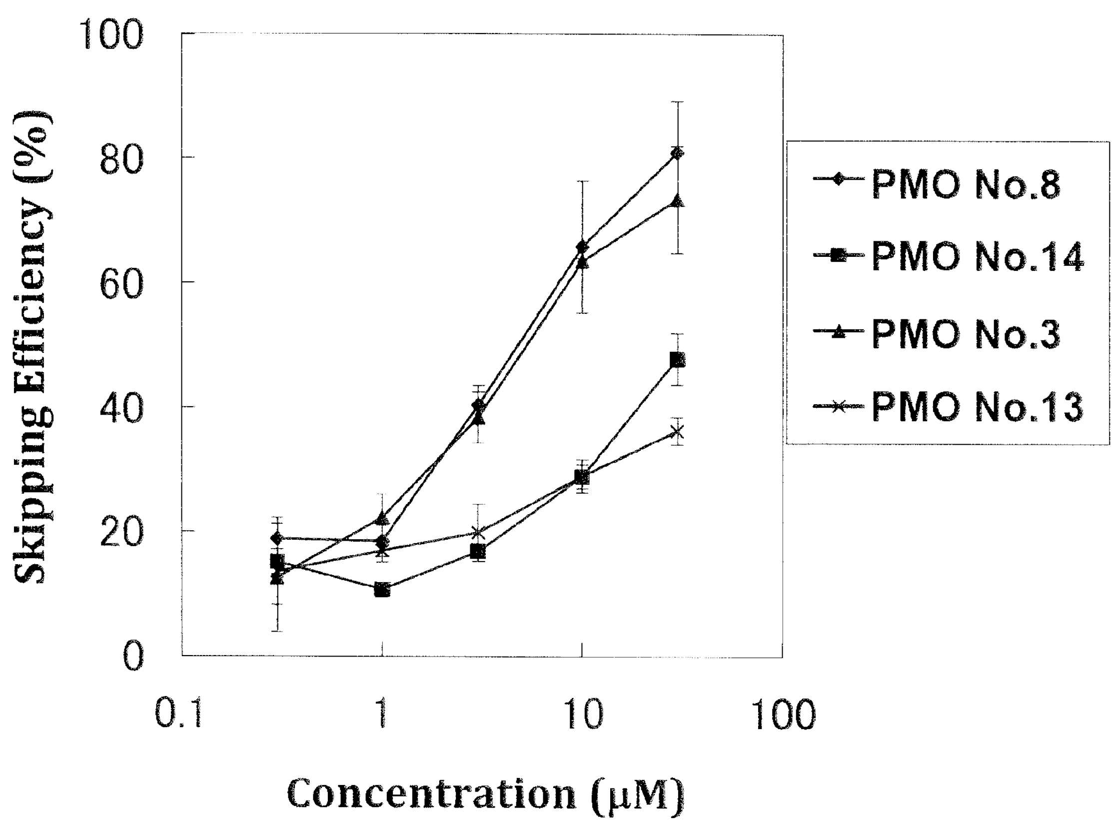

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 19



100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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- *Org. Lett.,* 2004, vol. 6 (15), 2555-2557 **[0131]**

EXHIBIT AP

Europäisches Patentamt
European Patent Office
Office européen des brevets

Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5	Grant of European patent, and examination of the application under Article 94, are hereby requested.	☒
5.1	The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2))	☐
	Procedural language:	en
	Description and/or claims filed in:	en
6	Applicant's or representative's reference	PEP02753ABE
	Applicant 1	
7-1	Name:	Nippon Shinyaku Co., Ltd.
8-1	Address:	14, Kisshoin Nishinosho Monguchicho Minami-ku Kyoto-shi Kyoto Kyoto 601-8550 Japan
10-1	State of residence or of principal place of business:	Japan
	Applicant 2	
7-2	Name:	National Center of Neurology and Psychiatry
8-2	Address:	1-1, Ogawa-Higashi-cho 4-chome Kodaira-shi Tokyo Tokyo 187-8551 Japan
10-1	State of residence or of principal place of business:	Japan
14.1	The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC.	☐

Representative 1

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	Company:	Lederer & Keller Patentanwälte Partnerschaft mbB
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17-1	Telephone:	+49 89 21 23 99 0
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17-1	E-mail:	info@lederer-keller.de

Representative 2

15-2	Name:	Best, Dr. Michael
	Same address as Representative 1	☒

Representative 3

15-3	Name:	TEIPEL, Dr. Stephan
	Same address as Representative 1	☒

Representative 4

15-4	Name:	KALHAMMER, Dr. Georg
	Same address as Representative 1	☒

Inventor(s)

23 Inventor details filed separately ☒

24 Title of invention

Title of invention:	ANTISENSE NUCLEIC ACIDS
Translation of title into French:	ACIDES NUCLÉIQUES ANTISENS
Translation of title into German:	ANTISENSE-NUKLEINSÄUREN

25 Declaration of priority (Rule 52)

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	JP	01.09.2010	ap	2010-196032	☐

25.2 This application is a complete translation of the previous application ☐

25.3 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☒

Application number of earlier application: EP11821996.3

Date of Filing (Art. 80/Rule 40 EPC): 31 August 2011

27.1 This divisional application is of the following generation: ☒ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 or subsequent

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 4

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No.

31 Designation of contracting states

All the contracting states party to the EPC and valid for the parent application at the time of filing of this divisional application are deemed to be designated (see Article 76(2)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s):

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description	☒
The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25	☒
The sequence listing is attached in PDF format	☐
38.3 The Office is requested to add to the dossier on the European patent application, in electronic form and for search purposes only (i.e. not as part of the description), a copy of the Standard-compliant sequence listing filed for the earlier application mentioned in Section 27. It is hereby declared that the sequence listing does not extend beyond the content of the divisional application as originally filed.	☒

Further indications

39 Additional copies of the documents cited in the European search report are requested	
Number of additional sets of copies:	
40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested	☒
Application or publication number of earlier search report:	11821996.3

42 Payment

Mode of payment	Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated on the fees section below.

Currency:	EUR
Deposit account number:	28000381
Account holder:	Lederer & Keller

43 Refunds

Fees	Factor applied	Fee schedule	Amount to be paid
001 Filing fee - EP direct - online	1	120.00	120.00
002 Fee for a European search - Applications filed on/after 01.07.2005	1	1 285.00	1 285.00
015 Claims fee - For the 16th to the 50th claim	0	235.00	0.00
015e Claims fee - For the 51st and each subsequent claim	0	580.00	0.00
033 Renewal fee for the 3rd year	1	465.00	465.00
034 Renewal fee for the 4th year	1	580.00	580.00
035 Renewal fee for the 5th year	1	810.00	810.00
501 Additional filing fee for the 36th and each subsequent page	51	15.00	765.00
Total:		EUR	4 025.00

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	Application text.pdf Description; 4 claims; 19 figure(s); abstract	SPECEPO-1.pdf
B-2	Sequence listings, ASCII	G12-0074_Seq Listing.txt	SEQLTXT.txt

44-C Other documents		Original file name:	System file name:
C-1	Search Report	Search results.pdf	OTHER-1.pdf
C-2	Additional Sheet	Additional Sheet.pdf	OTHER-2.pdf

45	General authorisation:	

46 Signature(s)

Place: **München**

Date: **11 December 2015**

Signed by:	**Günter Keller 6097**
Representative name:	**Günter KELLER**
Capacity:	**(Representative)**

Claims for Divisional Application

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of: the 31st to the 55th, the 32nd to the 53rd, the 32nd to the 61st, the 33rd to the 54th, the 33rd to the 57th, the 34th to the 58th, the 35th to the 59th, the 36th to the 53rd, the 36th to the 55th, the 36th to the 57th, the 36th to the 60th, or the 37th to the 61st nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

2. The antisense oligomer according to claim 1, which is an oligonucleotide.

3. The antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

4. The antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

The examination is being carried out on the **following application documents**

Description, Pages

1-65 as originally filed

Sequence listings, SEQ ID NO

1-123 as originally filed

Claims, Numbers

1-4 as originally filed

Drawings, Sheets

1/19-19/19 as originally filed

***

1. Documentation

1.1 Reference is made to the following document; the numbering will be adhered to in the rest of the procedure.

D1 POPPLEWELL L J ET AL: "Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials", NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB,
vol. 20, no. 2, 1 February 2010 (2010-02-01), pages 102-110,
XP026878306,
ISSN: 0960-8966

2. Article 76(1) EPC

2.1 It appears from the translated claims of the parent application that part of the claimed subject-matter was originally not claimed. However, the sequences from position 36-60 and 37-61 have been disclosed in the sequence listing. As Article 76(1) EPC refers to the content of the earlier application, the presently claimed subject-matter is considered to meet the requirements.

3. Support, Article 84 EPC

3.1 By comparing the claimed oligonucleotides and table 2 it was seen that several (four) sequences have not been made into oligonucleotides and are therefore also not

tested in any of the examples. In the technical field of antisense molecules a single nucleotide change can have a dramatic effect on its suitability. Therefore, lack of technical support for these oligonucleotides is raised.

4. Novelty, Article 54 EPC

4.1 The claimed oligonucleotides in as far as supported are new.

5. Inventive step, Article 56 EPC

5.1 For the parent application a positive inventive step assessment was provided. This was based on the clear improved exon skipping caused by the molecules claimed over one known from the prior art. In the present situation other molecules are claimed, some of them not even having been tested. Therefore, an improvement in efficiency cannot be seen as part of the problem to be solved.

5.2 The problem and solution assessment for the non-supported oligos uses D1 as closest prior art. D1 discloses several PMO's able to cause exon 53 skipping. The H53A30/1 variant is considered the closest and has extensive overlap with the claimed sequences. The differences of the present sequences over the oligo of D1 is that they are smaller in size. The technical effect of this difference is unknown as comparative data is not present for all claimed sequences.

5.3 The problem to be solved is the provision of alternative exon 53 skipping oligonucleotides.

5.4 The solution to said problem is obvious. The skilled person starting from H53A30/1 can make many variants in position and size with the expectation of making alternatives. For making improvements he would have had only the hope to succeed. The subject-matter of claims 1-4 is not inventive.

LEDERER & KELLER
Patentanwälte Partnerschaft mbB
European Patent and Trademark Attorneys

EPO - Munich
78
10. Nov. 2016

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Your reference

Our reference
PEP02753ABE

10 November 2016
K/sh

Re.: European Patent Application No. 15199455.5-1401
Nippon Shinyaku Co., Ltd. / National Center of Neurology and Psychiatry

This is in response to the communication pursuant to Rule 69 EPC and invitation pursuant to Rule 70a(1) EPC dated May 17, 2016:

With the enclosed payment order the following fees are paid for the above-referenced European patent application:

- the examination fee
- the designation fee for all European contracting states.

With regard to the comments attached to the extended European search report dated March 16, 2016 we propose the following amendments and submit arguments:

I. Amended set of claims

We submit in the enclosure an amended set of claims in correction mode and as clean copy whereby the claims are based on the claims as filed with the present divisional application.

The amended set of claims is focused on an antisense oligomer consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotide from the 5' end of the 53rd exon in the human dystrophin gene.

The antisense oligonucleotide is disclosed at several places in the specification as originally filed. In Table 7 (page 53) the antisense oligomer H53_36-60 is mentioned and the corresponding SEQ ID Number is 57.

From the experimental part, whereby the results are shown in Figures 13, 16 and 17, it can be learned that this oligomer has a high skipping efficiency whereby an outstanding effect is shown in Figure 13.

In view of the comments provided in the Annex to the extended European search report, in particular under section 2, it is assumed that the enclosed set of claims is allowable in view of Article 123(2) EPC. The Examining Division is respectfully requested to confirm.

The objections as raised under Article 84 EPC are overcome by the proposed amendment.

II. Inventive step

The novelty of the claimed oligonucleotides has already been acknowledged (section 4). Concerning the presence of an inventive step the Examining Division has considered document D1 (Popplewell et al.) as closest prior art.

According to the Examining Division the H53A30/1 variant is considered to be the closest embodiment which has an overlap with the claimed sequences. This phosphorodiamidate morpholino oligomer (PMO) is designated in Table 1 as "**G**". This PMO-G is considered to be the most efficient PMO (page 104, right column, second paragraph).

We submit, however, that the claimed antisense oligomer of the present application is not rendered obvious by D1 since there is no motivation to change the disclosed sequence in D1. Moreover, the claimed antisense oligomer has a superior skipping activity compared with the PMO-G as disclosed in D1.

Unfortunately the specification as filed does not contain a direct comparison of the oligomers as disclosed in D1 and the antisense oligomer according to the present claims. There is, however, an indirect comparison which clearly shows that the claimed oligomer has a superior activity.

In the specification of the present application on page 39, Table 2, there is a PMO No. 12 which has a target sequence in exon 53 of nucleotide 30-59. This oligonucleotide is represented by SEQ ID NO:39. This sequence is also mentioned under PMO No. 15 (page 39, 2nd PMO from the bottom). This PMO corresponds to H53A30/1 as disclosed in D1.

In Figures 2 and 3 of the present application it is shown that SEQ ID NO:11 corresponding to H53_32-56 (SEQ ID NO:11) corresponding to PMO No. 3 has a higher activity than the oligomer disclosed by Popplewell et al.

Furthermore, H53_36-60 (SEQ ID NO:57) has a higher skipping activity than H53_32-56 (SEQ ID NO:11) as shown in Figures 16 and 17.

From the above explanations it is clear that H53_36-60 (SEQ ID NO:57) has higher skipping activities than the best oligomer disclosed by Popplewell et al. This could not be expected from the prior art. Therefore, it is respectfully submitted that the enclosed set of claims should be considered as inventive.

III. Requests

The Examining Division is respectfully requested to acknowledge the admissibility of the enclosed set of claims. The adaptation of the specification can be effected upon short notice.

In case the Examining Division intends to raise further objections it is requested to issue a further communication or to arrange an informal interview either over the telephone or in personam with the undersigned attorney. As auxiliary measure oral proceedings according to Article 116 EPC are requested.

G. Keller

Dr. G. Keller

Enc.:
amended set of claims in correction mode
amended set of claims as clean copy
payment order

Zur Kasse
€220,-

EPA 15199455.5
Nippon Shinyaku Co., Ltd. et al.

Claims

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

2. The antisense oligomer according to claim 1, which is an oligonucleotide.

3. The antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

4. The antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

The examination is being carried out on the **following application documents**

Description, Pages

1-65 as originally filed

Sequence listings, SEQ ID NO

1-123 as originally filed

Claims, Numbers

1-4 received on 10-11-2016 with letter of 10-11-2016

Drawings, Sheets

1/19-19/19 as originally filed

***

1. Documentation

1.1 Reference is made to the following document; the numbering will be adhered to in the rest of the procedure.

D1 POPPLEWELL L J ET AL: "Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials", NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB, vol. 20, no. 2, 1 February 2010 (2010-02-01), pages 102-110, XP026878306, ISSN: 0960-8966

2. Amendments, Article 123(2) EPC

2.1 The present set of claims meets the requirements of Article 123(2) EPC.

3. Inventive step, Article 56 EPC

3.1 In the ESOP the search examiner raised objections for lack of inventive step. The applicant replied on 10.11.2016 bringing arguments in favor of an inventive step.

3.2 The examining division agrees that an improvement in exon skipping by the claimed oligo(s) over the closest prior art h53A30/1 of D1 is indirectly demonstrated. However looking further in table 1 of D1 it turns out that PMO designated H (H53A30/2) fully encompasses (33-62 versus 36-60) the presently claimed oligonucleotide. This compound has a 30% higher exon skipping efficiency than

compound G. There is no data on the technical effect of the difference between the claimed oligo and compound H. Therefore, the following analysis applies.

3.3 Closest prior art D1 discloses several PMO's able to cause exon 53 skipping. The H53A30/2 variant is considered the closest in sequence and comprises the claimed smaller oligonucleotide. The difference of the present sequence over the oligo of D1 is the smaller size and therefore different sequence. The technical effect of this difference is unknown.

3.4 The problem to be solved is the provision of alternative exon 53 skipping oligonucleotides.

3.5 The solution to said problem is the antisense oligonucleotide consisting of a sequence complementary to the 36-60th nucleotide from the 5' end of exon 53. The skilled person starting from D1 sees that compound H (H53A30/2) has the highest efficiency. He, therefore could and would make variants in attempts to further optimize the efficiency or find alternatives. Hereby, small deviations in position and size reduction will be performed. In doing so he not only could but would arrive at the presently claimed oligonucleotide without any inventive step. Minor improvements are also expected, it is only when substantial improvements are found that the reasonable expectation changes into "the hope to succeed". The subject-matter of claims 1-4 is not inventive.

3.6 It is clear that comparative data might be the only solution to demonstrate the presence of an inventive step.

4. Final remarks,

4.1 It is not at present apparent which part of the application could serve as a basis for a new, allowable claim. Should the applicant nevertheless regard some particular matter as patentable, an independent claim should be filed taking account of Rule 43(1) EPC. The applicant should also indicate how the subject-matter of the new claim differs from the state of the art and the significance thereof.

The examination is being carried out on the **following application documents**

Description, Pages

1-65 as originally filed

Sequence listings, SEQ ID NO

1-123 as originally filed

Claims, Numbers

1-4 received on 10-11-2016 with letter of 10-11-2016

Drawings, Sheets

1/19-19/19 as originally filed

***

1. Inventive step, Article 56 EPC

1.1 In the office action of 09.02.2017 the examining division argued against inventive step based on D1 and the problem to be solved as finding an alternative exon 53 skipping AON. It was reasoned that there would have been a reasonable expectation of success.

1.2 With letter of 01.08.2017 the applicant responds that D1 teaches away from using 25 mers and that there would not have been a reasonable expectation as a lower level of exon-skipping would be expected.

1.3 However, there are no comparative data. The claimed oligonucleotide could indeed be much lower in exon skipping activity (it is only required that it has such activity). But for the problem to be solved this is not relevant as it was defined as finding an "alternative", or maybe even better worded "a further exon 53 skipping" oligonucleotide. Therefore a skilled person having been tought by D1 that the region 33-65 is providing good exon skipping activity has a defined region in which alternative or further AONs can be designed. Using a smaller AON is considered by D1 as resulting in lower activity, but again that is not relevant for the problem to be solved.

1.4 The applicant is given a final opportunity to file supportive data showing an improved activity. If this cannot be the case, the inventive step objection will be

maintained and the next communication will be a summons for oral proceedings. The proceedings will be held in The Hague.

HGF

European Patent Office
PB 5818 Patentlaan 2
2280 HV RIJSWIJK (ZH)
Netherlands

16 March 2018

Our ref: P251891EPD1 / TIM

By CMS

Dear Sirs

European Divisional Patent Application No 15199455.5 based on 11821996.3
Antisense Nucleic Acid
Nippon Shinyaku Co., Ltd. & National Center of Neurology and Psychiatry

In response to the Communication dated 8th September 2017 we file herewith:

- Experimental Report

Response to Objections
Our comments on the examiner's objections in the Examination Report are as follows:

In response to the Examiner's comments regarding comparative data, the applicant submits herewith an Experimental Report showing that the claimed oligomer has a higher skipping efficiency than the closest embodiment described in the prior art, *i.e.* H53A30/1 of D1.

In the Experimental Report, the skipping activity of the claimed oligomer "H53_36-60" is compared with "H53_33-62" (=H53A30/1 of D1), where "H53_36-56" is used as a control for comparison.

In the results of experiment 1, the activity of the claimed oligomer is shown as 45.5% against 68.0% of activity for the control.

In contrast, in the results of experiment 2, the activity of "H53_33-62" is shown as 32.7% against 76.2% of activity for the control.

The activities for the claimed oligomer "H53_36-60" and "H53_33-62" shown in the tables need to be corrected based on the activities of the control so that they can be directly compared.

When the activity of the control is adjusted to 100%, then the corrected activity for the claimed oligomer "H53_36-60" is:

Activity of "H53_36-60" per 100% of Control = 45.5% x (100%/68.0%) = 66.9%

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When the activity of the control is adjusted to 100%, then the corrected activity for "H53_33-62" is:

Activity of "H53_33-62" per 100% of Control = 32.7% x (100%/76.2%) = 42.9%

As such, the activity of "H53_36-60", 66.9%, is approximately 1.5-fold higher than that of H53_33-62", 42.9%.

The Experimental Report confirms the improved activity over H53A30/1 of D1, which is consistent with the data shown in the original specification as the applicant discussed in its previous response dated 10th November 2016.

Considering the fact that D1 teaches that that higher activities can be achieved (only) by using longer oligonucleotides (see page 108, right column, lines 1-9 from the bottom), the effect of the claimed oligomer, which is 25mer, having a higher skipping activity over H53A30/1 of D1, which is 30mer, goes against said teaching and thus is surprising.

It is respectfully submitted that the claims comply with the requirements of Article 56 EPC.

We believe that all of the examiner's objections have been addressed and we look forward to receiving a notice of intention to grant in due course. In the event that any matters remain outstanding, the examiner is invited to contact the writer by telephone in order that those issues can be speedily resolved. As a formal precautionary measure, in the event that the Examining Division is minded to refuse this application without further written procedure, we hereby request oral proceedings via video conference. Please ensure that an IP technology video conferencing facility is available at the time of any appointment of the proceedings. If the Examining Division is of the view that it would refuse the video conferencing request, please inform us of the reasons for the refusal in accordance with GL Part E, II, 11.1.1. The applicant reserves the right to request appearance at any oral proceedings in person and will notify the EPO if this is the case.

Yours faithfully

Kate Laura TAYLOR
Professional Representative
For and on behalf of HGF Limited
Association No. 145

The examination is being carried out on the **following application documents**

Description, Pages

1-65	as originally filed

Sequence listings, SEQ ID NO

1-123	as originally filed

Claims, Numbers

1-4	received on	10-11-2016	with letter of	10-11-2016

Drawings, Sheets

1/19-19/19	as originally filed

***

1. Inventive step, Article 56 EPC

1.1 The examining division has considered the additional technical information filed with your letter of 16.03.2018.

1.2 The data is in support of the presence of an inventive step. Which is hereby acknowledged.

2. Final remarks,

2.1 The claims are allowable. The applicant is therefore requested to bring the description into conformity with these claims; care should be taken during revision, especially of the introductory portion including any statements of problem or advantages, not to add subject-matter which extends beyond the content of the application as originally filed (Article 123(2) EPC).

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

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ROYAUME UNI

Date
11.07.19

Reference	Application No./Patent No.
P251891EPD1/KLT	15199455.5 - 1111 / 3018211
Applicant/Proprietor	
Nippon Shinyaku Co., Ltd., et al	

Decision to grant a European patent pursuant to Article 97(1) EPC

Following examination of European patent application No. 15199455.5 a European patent with the title and the supporting documents indicated in the communication pursuant to Rule 71(3) EPC (EPO Form 2004C) or in the information (EPO Form 2004W, cf. Notice from the EPO dated 8 June 2015, OJ EPO 2015, A52) dated 23.04.19 is hereby granted in respect of the designated Contracting States.

Patent No.	: 3018211
Date of filing	: 31.08.11
Priority claimed	: 01.09.10/JPA 2010196032
Designated Contracting States and Proprietor(s)	: AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR Nippon Shinyaku Co., Ltd. 14, Kisshoin Nishinosho Monguchicho, Minami-ku Kyoto-shi, Kyoto 601-8550/JP AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR National Center of Neurology and Psychiatry 1-1, Ogawa-Higashi-cho, 4-chome Kodaira-shi Tokyo 187-8551/JP

This decision will take effect on the date on which the European Patent Bulletin mentions the grant (Art. 97(3) EPC).

The mention of the grant will be published in European Patent Bulletin 19/32 of 07.08.19.

Registered letter
EPO Form 2006A 07.15 (04/07/19)

to EPO postal service: 05.07.19

Date

Application No. 15199455.5

Examining Division

Kools, Patrick Lejeune, Robert Andres, Serge

ANMERKUNG ZUR ENTSCHEIDUNG ÜBER DIE ERTEILUNG
EINES EUROPÄISCHEN PATENTS (EPA Form 2006)

1. **EPA Informationsbroschüre "Nationales Recht zum EPÜ"**
Diese Broschüre enthält nützliche Informationen zu den formalen Erfordernissen und den Handlungen, die vor den Patentbehörden der Vertragsstaaten vorzunehmen sind, um Rechte in diesen Staaten zu erlangen. Da diese Handlungen einem ständigen Wandel unterworfen sind, sollte immer nur die neueste Ausgabe der Broschüre benutzt werden. Nachträgliche Informationen werden im Amtsblatt veröffentlicht.

2. **Übersetzung der europäischen Patentschrift nach Artikel 65 (1) des Europäischen Patentübereinkommens**
Sie werden erneut darauf hingewiesen, dass bestimmte Vertragsstaaten nach Artikel 65 (1) EPÜ eine Übersetzung der europäischen Patentschrift verlangen; hierauf wird in der Mitteilung gemäß Regel 71 (5) EPÜ verwiesen. Die Nichteinreichung dieser Übersetzung kann zur Folge haben, dass das Patent in dem betreffenden Staat/in den betreffenden Staaten als von Anfang an nicht eingetreten gilt. Weitere Einzelheiten entnehmen Sie bitte der oben genannten Broschüre.

3. **Zahlung von Jahresgebühren für europäische Patente**
Nach Artikel 141 EPÜ können "nationale" Jahresgebühren für das europäische Patent für die Jahre erhoben werden, die an das Jahr anschließen, in dem der Hinweis auf die Erteilung des europäischen Patents im "Europäischen Patentblatt" bekanntgemacht wird. Weitere Einzelheiten entnehmen Sie bitte der oben genannten Broschüre.

NOTE RELATING TO THE DECISION TO GRANT A
EUROPEAN PATENT (EPO Form 2006)

1. **EPO Information Brochure "National law relating to the EPC"**
This brochure provides useful information regarding formal requirements and the steps to be taken before the patent authorities of the Contracting States in order to acquire rights in those states. Since the necessary steps are subject to change the latest edition of the brochure should always be used. Subsequent information is published in the Official Journal.

2. **Translation of the European patent application under Article 65(1) of the European Patent Convention**
Your attention is again drawn to the requirements regarding translation of the European patent specification laid down by a number of Contracting States under Article 65(1) EPC, to which reference is made in the communication under Rule 71(5) EPC. Failure to supply such translation(s) may result in the patent being deemed to be void "ab initio" in the State(s) in question. For further details you are recommended to consult the above-mentioned brochure.

3. **Payment of renewal fees for European patents**
Under Article 141 EPC "national" renewal fees in respect of a European patent may be imposed for the years which follow that in which the mention of the grant of the European patent is published in the "European Patent Bulletin". For further details you are recommended to consult the above-mentioned brochure.

REMARQUE RELATIVE A LA DECISION DE DELIVRANCE
D'UN BREVET EUROPEEN (OEB Form 2006)

1. **Brochure d'information de l'OEB "Droit national relatif à la CBE"**
Cette brochure fournit d'utiles renseignements sur les conditions de forme requises et sur les actes à accomplir auprès des offices de brevet des Etats contractants aux fins d'obtenir des droits dans les Etats contractants. Etant donné que les actes indispensables sont susceptibles de modifications, il serait bon de toujours consulter la dernière édition de la brochure. Toute information ultérieure est publiée au Journal Officiel.

2. **Traduction du fascicule du brevet européen en vertu de l'article 65(1) de la Convention sur le brevet européen**
Votre attention est de nouveau attirée sur l'obligation faite par certains Etats contractants, en vertu de l'article 65(1) CBE, de fournir une traduction du fascicule du brevet européen, à laquelle il est fait référence dans la notification établie conformément à la règle 71(5) CBE. Si la(les) traduction(s) n'est(ne sont) pas fournie(s), le brevet européen peut, dès l'origine, être réputé sans effet dans cet(ces) Etat(s). Pour plus de détails, nous vous renvoyons à la brochure susmentionnée.

3. **Paiement des taxes annuelles pour le brevet européen**
Conformément à l'article 141 CBE des taxes annuelles "nationales" dues au titre du brevet européen peuvent être perçues pour les années suivant celle au cours de laquelle la mention de la délivrance du brevet européen est publiée au "Bulletin européen des brevets. Pour plus de détails, nous vous renvoyons à la brochure susmentionnée.

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
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GERMANY

Questions about this communication ?
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Date
19.08.19

Reference	Application No./Patent No.
P251891EPD1/KLT	15199455.5 - 1111 / 3018211
Applicant/Proprietor	
Nippon Shinyaku Co., Ltd., et al	

Transmission of the certificate for a European patent pursuant to Rule 74 EPC

The certificate for a European patent is herewith transmitted.

The European patent specification can be downloaded from the EPO publication server **https://data.epo.org/publication-server/** (see OJ EPO 2005, 126).

Note:
A corrected title page of the European patent specification will be published, if the bibliographic data have been changed after completion of the technical preparations.

For the Examining Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

SRPT-VYDS-0226522

D YOUNG&CO

OPPOSITION

TO

EP3018211B

Nippon Shinyaku Co., Ltd.

AND

National Center of Neurology and Psychiatry

BY:

Sarepta Therapeutics, Inc.

215 First Street

Cambridge, MA 02142

USA

D YOUNG&CO

Contents

EXTENT OF OPPOSITION

EP3018211B (hereinafter '**211**) is opposed in its entirety for all designated states.

REQUESTS

We request that '211 be revoked in its entirety.

If anything other than complete revocation is envisaged, we request Oral Proceedings. This request is ongoing and applies in respect of any matter on which we have not been heard.

THE PATENT

'211 was filed as EP15199455.5, a divisional of EP11821996.3, which was filed as PCT/JP2011/70318 on 31 August 2011 and published as WO2012/029986 on 8 March 2012.

It claims priority from Japanese patent application JP2010196032 ("P1") filed on 1 September 2010.

However, as will be detailed below, the claimed subject-matter of '211 extends **beyond** the content of both the divisional application as filed and the parent application as filed. **Therefore, the claimed subject-matter is entitled only to the date the <u>amendments</u> were filed, namely 10 November 2016**.

D YOUNG&CO

DOCUMENTS CITED

D1 – EP2612917A

D2 – Oxford Dictionary of Biochemistry and Molecular Biology, Oxford University Press, revised edition, 2000

D3 – WO 2010/048586

D4 – US 2010/0168212

D5 – L.J. Popplewell et al., *Neuromuscular Disorders*, **2010**, 20(2), 102-110

D6 – WO 2014/153240

D7 - US 6784291

D8 - US 2010/0130591

D9 - WO 2004/083432

D10 – A. Aartsma-Rus et al., "Functional Analysis of 114 Exon-Internal AONs for Targeted DMD Exon Skipping: Indication for Steric Hindrance of SR Protein Binding Sites," Oligonucleotides (2005) 15(4): 284-297

D11 - WO 2006/000057

D12 - US 2007/0082861

D13 - WO 2011/057350

D1 is the English translation of the parent PCT application as published in accordance with Article 153(4) EPC. Although D1 published on 10 July 2013, the relevant publication date is that of the WO publication, namely 8 March 2012.

D2 is cited as evidence of the normal meaning of the word "complementary" in molecular biology and biochemistry.

D13 was published under the PCT on 19 May 2011, before the PCT filing date of the opposed patent. For the reasons explained in detail herein, as the opposed patent is not entitled to claim priority from P1 – thereby meaning that D13 forms prior art to the opposed patent under Article 54(2) EPC.

All of the other documents were published before the priority date P1 – thereby meaning that they all form prior art to the opposed patent under Article 54(2) EPC irrespective of whether the claim to priority is valid.

CLAIMED SUBJECT-MATTER OF THE OPPOSED PATENT

The granted claim set is reproduced in full in Annex 1.

There is only one independent claim, claim 1, which recites as follows:

1. *An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene,* ***consisting of*** *a nucleotide sequence* ***complementary to the 36th to the 60th nucleotides*** *from the 5' end of the 53rd exon in the human dystrophin gene.*

In this regard, the Opposition Division's attention is drawn to the following features of the claim.

Base sequence

Firstly, the term *"consisting of"* takes its usual closed meaning – in other words, that the sequence of the oligomer has the recited bases **and no others**.

The claim therefore limits the nucleotide sequence to a nucleotide sequence **complementary to the 36th to the 60th nucleotides** of exon 53. It can therefore be understood to have 25 bases.

The normal meaning of the term *"complementary"* in molecular biology and biochemistry in relation to an oligonucleotide chain is that the base sequence of the oligonucleotide chain is a complementary base sequence to the sequence of the target polynucleotide chain.

D2 confirms that the term "complementary base sequence" means *"a sequence in a polynucleotide in which* ***all*** *the bases are able to form base pairs with a sequence of bases in another polynucleotide chain".*

The DNA base sequence of exon 53 of the human dystrophin gene is disclosed as SEQ ID NO: 1 in '211. The 36^{th} to 60^{th} nucleotides thereof (read from 3' to 5' as this is the target sequence) have the base sequence CAACGGAGGCCAAGACTTCCACAAG.

This means that, under Watson-Crick base pairing rules (A pairing to T or U, and G to C), the normal meaning of the base sequence of the oligonucleotide chain **complementary** to it would be **GTTGCCTCCGGTTCTGAAGGTGTTC** or **GUUGCCUCCGGUUCUGAAGGUGUUC**.

However, paragraph [0026] of the granted specification indicates that the term "complementary" is intended by the patentee to have a **broader** meaning, as follows:

"As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does ***not only*** *refers to a nucleotide sequence* ***100% complementary to the target*** *nucleotide sequence but also refers to a complementary nucleotide sequence that* ***may contain, <u>for example</u>, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence****."*

This paragraph of the description therefore **does not restrict** the number of **non-complementary** bases.

In view of this, claim 1 of '211 should be interpreted such that the **base sequence** of the antisense oligomer may either be a 100% complementary to the target sequence, or may differ by **an unknown number** of non-complementary bases.

Antisense chemistry

Claim 1 of '211 only recites the nature of the base sequence. In contrast, it does **not** limit in any way the **chemistry** of the **backbone** of the antisense oligomer. Consequently, it covers **all** known antisense chemistries, including but not limited to the following:

- natural (RNA/DNA) oligomers having ribose or deoxyribose base-bearing subunits bonded together by phosphate intersubunit linkages;
- morpholino oligomers having morpholine base-bearing subunits, in particular phosphorodiamidate morpholino oligomers (PMOs) wherein the morpholine base-bearing subunits are bonded together by phosphorodiamidate intersubunit linkages
    - Evidence that the claims cover morpholino, in particular PMO, oligomers can be found at paragraphs [0056] (which indicates they are *"the oligomer of the present invention"*) to [0109]
- 2'O-methylphosphorothioate (2'-OMePS) oligomers having 2'O-methylribose base-bearing subunits bonded together by phosphorothioate intersubunit linkages.
    - Evidence that the claims cover (2'-OMePS) oligomers can be found at paragraph [0054] (which indicates they are *"the oligomer of the present invention"*)
- Peptide nucleic acids having amino acid base-bearing subunits bonded together by amide (peptide) bonds.
    - Evidence that the patentee intends the term to cover (2'-OMePS) oligomers can be found at paragraphs [0110] (which indicates they are *"the oligomer of the present invention"*) to [0111]

Both of the above issues, of course, mean the claims are essentially **<u>unlimited</u>** both in the nucleotide sequence and the antisense chemistry.

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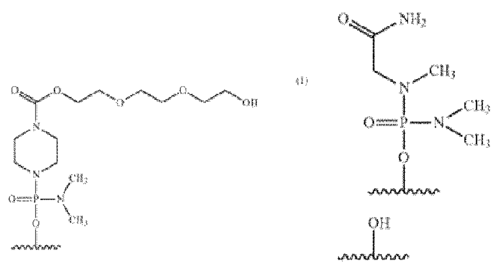
No definition of group at 5' end

It is noted that, in the description, '211 recites several groups which may be present at the 5' end of the molecule. Specifically, the following groups (1) to (3) are referred to in the detailed description (see, for example, the top of page 4 of '211):



However, the **claims** of '211 do **not** include any such limitation, and consequently cover antisense oligomers having **any** group at the 5' end of the oligomer.

The lack of any meaningful limitation in the claims (when interpreted in the light of the patentee's own definitions in the description) has consequences as regards added matter, sufficiency and inventive step as indicated below. In particular, the lack of a definition of the group at the 5'-end has consequences for inventive step in view of the data presented in '211, as explained in more detail below.

ADDED SUBJECT-MATTER – ARTICLES 123(2) & 76(1) EPC

Claim 1 extends beyond content of divisional and parent applications as filed

For the reasons outlined below, at least claim 1 of '211 constitutes **an unallowable intermediate generalisation** between the broadest disclosure and the Examples and therefore extends beyond both the content of the divisional application EP15199455.5 as filed, contrary to Article 123(2) EPC, and that of the parent application EP11821996.3 as filed, contrary to Article 76(1) EPC.

The description and drawings of the divisional application as filed are identical to those of the English translation of the parent PCT application as filed on EPO regional phase entry (with the exception that the claims of the parent as filed on EPO regional phase entry are reproduced as "items" at the end of the description of the divisional). In view of this, for ease of reference, both will be referred to generally in this Opposition as "the application as filed" unless there is a need to draw a distinction between the two.

It is established Boards of Appeal case law, and long established practice before the EPO, that intermediate generalisations violate Article 123(2) EPC. See Case Law of the Boards of Appeal (9th Edition, English version, II.E.1.9), pages 482 and 483, which summarises the key case law which prohibits intermediate generalisations under Article 123(2) EPC:

*"According to established case law (as summarised e.g., in **T 219/09** or **T 1944/10**), it will normally not be allowable to base an amended claim on the extraction of isolated features from a set of features originally disclosed only in combination, e.g. a specific embodiment in the description (**T 1067/97, T 714/00, T 25/03, T2095/12**).*

*Amended subject-matter that amounts to a generalisation of a particular embodiment disclosed in the original application but is still more specific than the original definition of the invention in general terms is often called an "intermediate generalisation" (see e.g. **T 461/05**, **T 191/04**; see also **T 2311/10**) and sometimes an "intermediate restriction" (see **T 461/05, T 879/09, T 2537/10**), Other decisions understand "intermediate generalisation" to refer to **an undisclosed – and thus unallowable - combination of selected features lying somewhere between an originally broad***

***disclosure and a more limited specific disclosure** (**T 1408/04**). An intermediate generalisation is different from a simple generalisation (as e.g. in **T 910/03**, **T 461/05**), since in the former case a definition of the invention in general terms forms part of the original disclosure (**T 461/05**)."*

In the case of divisional applications, the same principles are to be applied for determining whether subject-matter extends beyond the content of the earlier application as filed (**G 1/05**, OJ 2008, 271, point 5.1 of the Reasons – as cited in Case Law of the Boards of Appeal (9th Edition, English version, II.E.1.1), page 433. Therefore, all objections relating to contraventions of Article 123(2) EPC as regards the divisional as filed are equally applicable as contraventions of Article 76(1) EPC as regards the parent as filed.

As indicated above, Claim 1 of '211 reads as follows:

*"An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to **the 36th to the 60th nucleotides** from the 5' end of the 53rd exon in the human dystrophin gene."*

As noted above, this claim specifies the target sequences, but **not** the antisense chemistry.

It is acknowledged that the general paragraphs in the description which relate to the "disclosure of the invention", and specifically page 3, line 32 to page 4 line 10 (embodiment [1]) and page 7 line 31 to page 8, disclose in a generalised manner "oligomers of the present invention" consisting of antisense oligomers consisting of a nucleotide sequence complementary to the following nucleotides of exon 53:

31st to 53rd	32nd to 53rd	33rd to 53rd	34th to 53rd	35th to 53rd	36th to 53rd
31st to 54th	32nd to 54th	33rd to 54th	34th to 54th	35th to 54th	36th to 54th
31st to 55th	32nd to 55th	33rd to 55th	34th to 55th	35th to 55th	36th to 55th
31st to 56th	32nd to 56th	33rd to 56th	34th to 56th	35th to 56th	36th to 56th
31st to 57th	32nd to 57th	33rd to 57th	34th to 57th	35th to 57th	36th to 57th
31st to 58th	32nd to 58th	33rd to 58th	34th to 58th	35th to 58th	36th to 58th

No fewer than 36 target sequences within exon 53 ranging from the regions of 31st to the 55th to the 36th to the 58th nucleotides of exon 53 are disclosed in these general paragraphs. Antisense oligomers consisting of nucleotide sequences complementary to **these target sequences** can be considered disclosed in a generalised manner (i.e. **without** limitation of the antisense chemistry) in the application as filed.

In total contrast, there is **no** such **generalised** disclosure **anywhere** in the application as filed for an antisense oligomer consisting of a nucleotide sequence complementary to **the 36th to the 60th nucleotides** of exon 53. **None** of the general paragraphs in the application as filed disclose an antisense oligomer (of any antisense chemistry) consisting of a nucleotide sequence complementary to this target sequence.

The **sole basis** in the application as filed for an antisense oligomer targeting the 36th to 60th nucleotides of exon 53 is in **Test Example 6** – namely in Table 7 at page 53, in which an oligomer designated "H53_36-60" is disclosed as SEQ ID NO: 57 on page 54. It is **admitted** by the patentee, both in the basis table provided with the divisional application as filed on 11 December 2015 and in the response dated 10 November 2016, that this is the sole basis for claim 1.

However, in contrast to the sequences disclosed more generally in embodiment [1], the application as filed does **not** disclose either this oligomer or any of the other oligomers in Table 7 **in a generalised manner**. In contrast, page 53 lines 16 and 17, which describe the oligomers in Table 7, disclose that **all** of the antisense oligomers of Table 7 **are "2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA)**." (This term can be understood to read "2'-O-**methyl**-phosphorothioates").

This of course means that **all** of the Examples disclosed in Table 7 constitute a **combination of features**: a nucleotide sequence as disclosed therein **and** a 2'-O-methylphosphorothioate chemical backbone.

In contrast, as indicated above, claim 1 as currently on file is **completely silent** on the antisense chemistry, and therefore covers an antisense oligomer having **any** chemical backbone.

As is immediately apparent from the above, an amendment which introduces **only** the feature of the base sequence of SEQ ID NO: 57, but **not** the antisense chemistry with which it is disclosed **in combination**, constitutes **an intermediate generalisation** which violates Article 123(2) EPC. For the same reasons, it also extends beyond the content of the parent application as filed and violates Article 76(1) EPC.

As the patentee is of course aware, this objection has previously been raised in third party observations while the application was pending. However, the Examining Division **erred** in departing from its initial view (as indicated in the Communication dated 9 November 2018) that the above amendment violated Article 123(2) EPC and allowing the application to proceed to grant, for the following additional reasons.

The patentee's response dated 11 March 2019 cites an additional passage from Case Law of the Boards of Appeal, II.E.1.7 (corresponding to II.E.1.9 of the 9th Edition) relating to intermediate generalisations. In this regard, the Opponent has the following comments.

In T714/00 (Reasons 3.3) the Board reasoned as follows:

*"Extracting an isolated feature from an originally disclosed combination and using it for delimiting claimed subject-matter can only be allowable under the concept of Article 123(2) EPC if that feature is **not inextricably linked** with **further features of that combination.**"*

As the Opposition Division will understand from the above, an antisense molecule does not simply comprise a base sequence – the skilled person must consider **the whole molecule**, **including** the chemical backbone.

The patentee also relies heavily on T962/98 in its response dated 11 March 2019. The catchword of T962/98, which the patentee quotes, reads as follows:

*"There may exist situations where some characteristics taken from a working example may be combined with other features disclosed in a more general context without necessarily creating an objectionable intermediate generalization. However, under Article 123(2) EPC, such an intermediate generalization is only admissible if the skilled person can recognize **without any doubt** from the application as filed that*

those characteristics are not closely related to the other characteristics of the working example and apply directly and unambiguously to the more general context. In other terms, in order to be acceptable, this intermediate generalization must be the result of unambiguous information that a skilled person would draw from the review of the example and the content of the application as filed (cf. point 2.5)."

The patentee also refers to the summary of the experimental results of Test Example 6 as disclosed at page 57, lines 21-25 (as corrected) and alleges that this provides generalised teaching for an antisense oligomer that targets within the region from position 31-61 from the 5' end of exon 53.

We disagree. As indicated above, Test Example 6 of the application as filed discloses the antisense molecules of Table 7, **all** having a 2'-O-Me PS chemical backbone. The results described therein are therefore **specific** to **those molecules** and **cannot** be used to provide basis for a more generalised feature which is nowhere disclosed in the application as filed.

Put simply, and as stated above, Test Example 6 and Table 7 of the application as filed do **not** provide **generalised** teachings of AONs. In contrast to the AONs described above, which the patentee **defined** as *"oligomers of the present invention"*, nucleotide sequences complementary to **the 36th to the 60th nucleotides** of exon 53 are **not** part of that invention. The sole disclosure of such a compound targeting the 36th to the 60th nucleotides of exon 53 is in Test Example 6 and Table 7, where a single compound having the sequence: GUUGCCUCCGGUUCUGAAGGUGUUC, as defined in SEQ ID NO:57 **and** 2'OMePS chemistry is disclosed.

In addition, the patentee alleges that aim of the invention should be considered more generally as identification of the target regions within exon 53, and that the particular antisense chemistry is not singled out as being crucial. However, while this statement may be considered reasonable in respect of the antisense compounds referred to above disclosed more generally in embodiment [1] of the application as filed, it does not change the fact that the sole disclosure of an antisense oligomer targeting the 36th to 60th nucleotides of exon 53 is in Table 7 which specifically recites 2'-O-Me PS antisense oligomers.

In this regard, the specification repeatedly and specifically defines the **invention** using the defined term *"oligomers of the present invention"* as AONs consisting of sequences complementary to the 36 specified target sequences.

However, the AONs of Test Example 6 and Table 7 are not described as *"oligomers of the present invention."* Rather, the compounds of this example are simply described as *"antisense oligomers of 2'-O-methoxy-phosphorothioates."*

Nowhere in Test Example 6 and Table 7 does the patentee use the term *"oligomers of the present invention"*. It is **only** with respect to the *"oligomers of the present invention"* (i.e. AONs complementary to the 36 target sequences) that the chemical backbones are disclosed in a generalised manner.

The patentee specifically and intentionally limited that which they considered their invention to the *"oligomers of the present invention"* consisting of a nucleotide sequence that is complementary to the 36 target sequences. The AON "H53_36-60" in Test Example 6 and Table 7 does not fall within that invention, and therefore claiming it in a generalised manner violates Article 123(2) EPC.

Finally, the correction of the translation error on page 57 of the translated application as referred to in the response dated 11 March 2019 (namely, *"exons 31-61"* to *"positions 31-61"*) is **completely irrelevant** to the issue that Test Example 6 and Table 7 do not provide a generalised disclosure of AONs targeting the 36th to 60th nucleotides of exon 53. It does not change the fact that Test Example 6 only discloses H53_36-60 with the sequence of SEQ ID NO: 57 and 2'-OMe-PS chemistry.

Summarising, for all of the reasons indicated above, Claim 1 of '221 represents an unallowable intermediate generalisation having no basis in the application as filed, and therefore contravenes Article 123(2) and 76(1) EPC.

At least by their dependency on claim 1, claims 2-4 also contravene Article 123(2) and 76(1) EPC.

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LACK OF PRIORITY – ARTICLE 87 EPC

Subject-matter of claim 1 is not disclosed in P1

It is noted that no English translation of P1 is currently on file before the EPO. However, an inspection of the original Japanese of P1 clearly indicates that Test Example 6 and Table 7 of '211, which the Patentee admits is sole basis for the subject-matter of claim 1, is not disclosed anywhere in P1.

Therefore, **none** of the specific antisense compounds listed in Table 7, in particular, the specific 2'OMe-PS antisense compound having SEQ ID NO: 57, are disclosed anywhere in D1.

Therefore, Claim 1 of '211, and all claims dependent thereon, cannot validly claim priority from P1. Even if the Opposition Division disagrees with the additional arguments set out below regarding the date to which the claimed subject-matter is entitled, '211 is still only entitled to its PCT filing date of 31 August 2011.

Filing date to which the application is entitled is more than a year after P1

As detailed above, the claimed subject-matter is entitled only to the date the amendments were filed, namely 10 November 2016.

This is, of course, more than a year after the filing date of P1, namely 1 September 2010.

Therefore, for this additional reason, Claim 1 of '211, and all claims dependent thereon, cannot validly claim priority from P1.

NOVELTY – ARTICLE 54 EPC

Lack of novelty in view of D1

As detailed above, the claimed subject-matter of '211 is entitled only to the date the amendments were filed, namely 10 November 2016.

D1 was published before this filing date.

D1 discloses (as SEQ ID NO: 57) the sequence H53_36-60, which is an antisense oligomer, consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene, having a 2'-O-Me PS chemical backbone.

This causes skipping of the 53rd exon in the human dystrophin gene – see Test Example 7 and in particular the results shown in Figures 16 and 17 for H53_36-60.

Therefore, claim 1 lacks novelty in view of D1.

The features of claims 2-4 of '211 are also disclosed in D1, as follows:

- Claim 2 – embodiment [2] – page 3 line 27
- Claim 3 – embodiment [3] – page 3 lines 28-29
- Claim 4 – embodiment [4] – page 3 lines 30-32

Therefore, all claims of '211 lack novelty in view of D1.

Lack of novelty in view of D6

For the same reasons as detailed above, in view of the date to which the claimed subject-matter is entitled, D6 is also citable for novelty against '211.

D6 discloses (as SEQ ID NO: 1) the sequence H53A(36+60) – see D6, page 65, Example 2. This is an antisense oligomer, consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene, having a phosphorodiamidate morpholino (PMO) chemical backbone.

This AON causes skipping of the 53rd exon in the human dystrophin gene – see D7, page 70, Example 7 and in particular the results shown in Figures 3 and 4 for oligomer H53A(+36+60).

Therefore, claim 1 of '211 lacks novelty in view of D6.

INVENTIVE STEP – ARTICLE 56 EPC

In the unlikely event that the independent claims are deemed novel, the claims additionally lack inventive step for the reasons cited below.

Lack of inventive step over D5

D5 can be considered the closest prior art for the determination of inventive step. D5 discloses a number of PMOs targeting exon 53 of which at least 12 target regions that overlap with that of the instant claims. We further note that D8-D13 also disclose a number of AONs that target exon 53. For example, D8-D13 disclose at least 29 AONs that target regions within exon 53 that overlap the claim AONs. Accordingly, the claims lack an inventive step over D8-D13 as well.

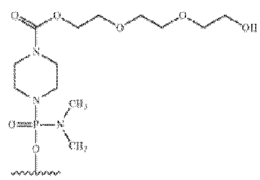
D5 was cited (as D1) by the Examining Division during prosecution. However, the Examining Division **erred** in departing from its initial opinion (as expressed in the Search Opinion dated 16 March 2016 and its subsequent Communications dated 9 February 2017 and 8 September 2017), for at least the reasons outlined below.

In its response dated 16 March 2018, the patentee provided comparative data between the oligomer "H53_36-60" as against "H53_33-62" (which is stated to be the antisense compound H53A30/1 of D3). Specifically, Patentee provided a description of the tested oligomers consisting of:

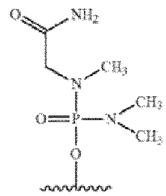
> **H53_36-60:** 5′- GTTGCCTCCGGTTCTGAAGGTGTTC -3′; corresponding to SEQ ID NO: 57 of the present application, and complementary to the 36th to the 60th nucleotides from the 5′ end of the human dystrophin gene's 53rd exon; and
> **H53_33-62:** 5′- CTGTTGCCTCCGGTTCTGAAGGTGTTCTTG-3′; corresponding to H53A30/2 of D1, and complementary to the 33rd to the 62nd nucleotides from the 5′- end of the human dystrophin gene's 53rd exon.
> **H53_36-56:** 5′- CCTCCGGTTCTGAAGGTGTTC -3′; corresponding to SEQ ID NO: 35 of the present application, and complementary to the 36th to the 56th nucleotides from the 5′ end of the human dystrophin gene's 53rd exon; and

Importantly, Patentee does not indicate many of the chemical features of the tested oligomers. Critically, Patentee fails to specify what functional group is present at the 5' end of the tested oligomers. Paragraph [0112], of '211 provides that *"[i]n an*

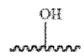
oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH".



(1)



(2)



(3)

We note that such groups were known and desirable in the prior art. For example, D7 states at column 7, lines 13-30:

"The solubility of the antisense compound, and the ability of the compound to resist precipitation on storage in solution, can be further enhanced by derivatizing the oligomer with a solubilizing moiety, such as a hydrophilic oligomer, or a charged moiety, such as a charged amino acid or organic acid. The moiety may be any biocompatible hydrophilic or charged moiety that can be coupled to the antisense compound and that does not interfere with compound binding to the target sequence. The moiety can be chemically attached to the antisense compound, e.g., at its 5' end, by well-known derivatization methods. One preferred moiety is a defined length oligo ethylene glycol moiety, such as ***triethylene glycol****, coupled covalently to the 5' end of the antisense compound through a* ***carbonate linkage, via a piperazine linking group forming a carbamate linkage with triethylene glycol****, where the second piperazine nitrogen is coupled to the 5'-end phosphorodiamidate linkage of the antisense."*

Here, D7 describes the moiety of Group (1) of the instant disclosure.

The description further provides (Test Example 7 – see '211 pages 34 and 35) that oligomers of the present invention having 5' end Group (3) (PMOs 8 and 14 – see Table 2) were superior to oligomers of the present invention having the same nucleotide sequence but having 5' end Group (1) (PMOs 3 and 13). (See Figure 19.)

The inventors concluded (see paragraph [0204] and Figure 19) that *"[t]hese results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences."*

In contrast, nowhere do the Experimental Results submitted with the response of 16 March 2018 describe what the group at the 5' end of any of the tested oligomers are or even if there is a group at the 5' end that corresponds to those disclosed in the patent.

In fact, an examination of Figure 19 shows that PMOs having a Group (3) -OH at the 5' end (PMOs 8 and 3) showed about a 1.375-2.2 fold increase in exon skipping activity as compared PMOs having the same nucleobase sequence and a Group (1) moiety at the 5' end (PMOs 14 and 13). For example, at 10µmol dose, exon 53 skipping for the PMO No. 8 with a Group (3) -OH was approximately 65% whereas exon 53 skipping for PMO 14 having a Group (1) moiety was approximately 30%; a 2.17-fold difference. Similarly, exon 53 skipping for PMO 3 having a Group (3) -OH was about 62% whereas exon 53 skipping for PMO 13 having a Group (1) moiety was about 30%; a 2.07-fold difference.

In their response dated 16 March 2018, Patentee indicated that the exon 53 skipping of "H53_36-60" was "1.5-fold higher than that of H53_33-62…."However, at least because Patentee did not indicate which group was at the 5' end of "H53_36-60," "H53_36-56," or "H53_33-62," one cannot determine whether any differences in exon skipping is a result of the nucleobase sequence of the tested compounds **or** the 5' group of the tested compounds, whether it is one of Groups (1), (2), or (3) or another moiety.

Therefore, it is impossible to conclude that the effects described in the Experimental Results provided by the patentee in its response dated 16 March 2018 would be applicable to a corresponding antisense molecule having the same base sequence and **any** of the described 5' end groups. The comparative data do **not** therefore provide sufficient evidence that a technical effect is **associated with the difference from the closest antisense molecule** as disclosed in D5.

In the absence of any evidence of such a technical effect, the objective problem can only be formulated as providing an **alternative** to the antisense molecules of D5.

For the reasons previously stated in the Communications, this is entirely obvious over D5. Therefore, the subject-matter of Claim 1 and all claims dependent thereon lacks inventive step over D5.

Lack of inventive step across entire scope of claims

The patent purports to solve the problem of providing further compounds which induce exon 53 skipping.

However, even if the Opposition Division were to decide that **some** subject-matter in the patent solves this technical problem, this still leaves a vast amount of subject-matter falling within the claims as not solving any technical problem, for the reasons set out below.

For example, as indicated above the patent claims at least provide **no limitation** on the chemistry of the backbone – as evidenced by the multiplicity of antisense chemistries referred to above which the patentee's own definition in the patent recites as *"the invention"* – nor any limitation on the group at the 5' end of the oligomer.

As remarked above, this of course means the claims are **practically unlimited** with respect to the antisense chemistry.

There is, of course, no evidence in the patent that any technical problem is solved across this unlimited scope.

This of course means that there is no evidence in the patent that an inventive step is exhibited **across the entire breadth of the claims**, as required by T939/92 and subsequent case law.

Hence, for this additional reason, claim 1 lacks inventive step.

DEPENDENT CLAIMS LACK INVENTIVE STEP

The remaining claims additionally lack novelty and/or inventive step, for the reasons outlined below:

Claim 2 is dependent on claim 1 and adds the feature that the antisense oligomer is an oligonucleotide.

This feature does not add anything novel or inventive as antisense oligonucleotides are disclosed in the documents filed herewith, for example in D5.

Claim 3 is dependent on claim 2 and adds the feature that the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

This feature does not add anything novel or inventive as such modified oligonucleotides are disclosed in the documents filed herewith, for example in D5 which discloses AONs having PMO chemistry.

Claim 4 is dependent on claim 3 and adds the feature that the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

This feature does not add anything novel or inventive as 2'OMe-PS oligonucleotides are disclosed in the documents filed herewith.

INSUFFICIENCY – ARTICLE 83 EPC

For a number of reasons as set out below, the patent is insufficiently disclosed in a number of fundamental respects. For these reasons at least, the entire patent therefore lacks sufficient disclosure, contrary to Article 83 EPC.

Lack of sufficient disclosure across entire scope of claims

Even if the Opposition Division were to consider that <u>some</u> subject-matter falling within the independent claims is sufficiently disclosed (which we refute), the claims still lack sufficient disclosure across their entire breadth, for the reasons set out below.

For example, as indicated above the patent claims at least provide **no limitation** on the chemistry of the backbone – as evidenced by the multiplicity of antisense chemistries referred to above which the patentee's own definition in the patent recites as *"the invention"* – nor any limitation on the group at the 5' end of the oligomer.

As remarked above, this of course means the claims are **practically unlimited** with respect to the antisense chemistry.

There is, of course, no evidence in the patent that antisense oligomers can be prepared, and would effective in exon 53 skipping, across this unlimited scope. This of course means that there is no enabling disclosure **across the entire breadth of the claims**, as required by T409/91 and subsequent case law.

Hence, for this additional reason, all claims contravene Article 83 EPC.

CONCLUSION

None of the claims as granted meets the requirements of the EPC.

Therefore the patent should be revoked in its entirety.

ANNEX 1 – GRANTED CLAIMS

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

2. The antisense oligomer according to claim 1, which is an oligonucleotide.

3. The antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

4. The antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

ANTISENSE NUCLEIC ACIDS

EUROPEAN PATENT 3 018 211

NIPPON SHINYAKU CO., LTD | NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY

O009698EP | OPPOSED BY JAMES POOLE LIMITED

1 INTRODUCTION AND REQUESTS

1.1 European patent 3018211 is hereby opposed by James Poole Limited under Article 99 and Rule 76 EPC, as set out in the enclosed Form 2300. This patent lies in the name of Nippon Shinyaku Co., Ltd. and National Center of Neurology and Psychiatry, and was granted from application 15199455.5. Opposition is made to the extent of all claims for all states.

1.2 The documents that are relied upon in this opposition are listed in section 6. The opponent may rely additionally upon documents that are mentioned in the patent itself.

1.3 Opposition is made under:

- Article 100(a) EPC because the claimed invention lacks novelty and an inventive step;
- Article 100(b) EPC because the patent does not disclose the invention in a manner sufficiently clear or complete for it to be performed by the skilled person; and
- Article 100(c) EPC because claim 1 includes subject-matter which extends beyond the content of the parent and divisional applications as filed.

1.4 Briefly, the main reasons for invalidity are as follows:

- The subject matter of claim 1 extends beyond the content of the application as originally filed, because it represents an inadmissible intermediate generalisation from the original disclosure of the claimed oligomer, which specified a particular chemistry. The skilled person would be well aware that the chemistry of the oligomer has an important role in determining the skipping efficiency of an antisense oligomer, yet there is no limitation as to any chemistry in granted claim 1.
- An antisense oligomer that targets positions 36-60 of exon 53, as recited in claim 1, is directly and unambiguously derivable from D2 (US 2010/0168212). The claimed antisense oligomers are therefore not novel.
- The claimed antisense oligomer is an obvious alternative to what is disclosed in the prior art. It would have been entirely obvious to the skilled person starting from the prior art to target positions 36-60 of exon 53 of the Duchenne muscular dystrophy (DMD) gene. D1 (Popplewell et al. (2010) *Neuromuscular Disorders*, 20:102-110) discloses an antisense oligomer that is only one base different compared to the claimed oligomers (PMO-A) and specifically suggests targeting the general region of exon 53, which encompasses both PMO-A and the claimed oligomer, namely positions 30-65. In addition, the patent includes data showing that the claimed antisense oligomer is objectively worse than the prior art oligomer PMO-A.
- In view of the data in the patent it is doubtful that antisense oligomers with <u>any</u> particular chemistry targeting positions 36-60 of exon 53 would necessarily produce effective skipping

efficiencies in any assay. As shown in the patent, minor changes in the backbone chemistry of an antisense oligomer can result in an oligomer that does not have an effective exon skipping efficiency. Furthermore, the level of exon skipping observed for a particular antisense oligomer is dependent on the system in which the oligomer is tested.

1.5 The opponent therefore requests revocation of the patent as a whole, under Article 101(2) EPC. In the event that the opposition division will not grant this request on the basis of the written proceedings, the opponent requests oral proceedings under Article 116 EPC.

2 ADDED SUBJECT MATTER

2.1 Claim 1 extends beyond the content of the application as originally filed, because its content represents an intermediate generalisation from the scope of the original disclosure.

Basis for claim 1 as provided by the applicants

2.2 The applicants provided the following basis for claim 1 in their reply to the search report, dated 10th November 2016.

> The antisense oligonucleotide is disclosed at several places in the specification as originally filed. In Table 7 (page 53) the antisense oligomer H53_36-60 is mentioned and the corresponding SEQ ID Number is 57.
>
> From the experimental part, whereby the results are shown in Figures 13, 16 and 17, it can be learned that this oligomer has a high skipping efficiency whereby an outstanding effect is shown in Figure 13.

It should be emphasised at this point that the applicants had little option but to highlight these sections of the specification in support, as Table 7, and Figures 13, 16 and 17 referenced in Example 6 provide the only places in the application as filed that mention H53_36-60 whatsoever.

2.3 Turning to Example 6 itself, this example actually discloses a large variety of RNA antisense oligomers, and all of these have a phosphorothioate-linked 2'-O-methyl modified base chemistry (2'OMePS). Between them, these oligomers target different regions of exon 53 of the DMD gene. Table 7 actually discloses 74(!) different oligomers in total. Certainly, no emphasis whatsoever is given to the antisense oligomer H53_36-60 which is cited as basis for the region of exon 53 recited in claim 1 of the opposed patent.

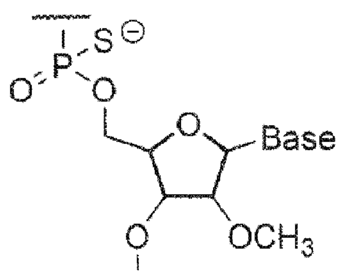
2.4 One first point to make is therefore that the applicants are selecting a single 2'OMePS RNA antisense oligomer from this very long list, and the oligomer selected does not even have the best exon skipping activity. In this respect, Figures 16 and 17 show that 2'OMePS RNA antisense oligomers targeting positions 32-61, 33-57, 34-58 and 35-59 work better than the claimed antisense oligomer (see Figure 17 of the application as filed discussed in section 4.21 below). The selection of a 2'OMePS RNA antisense oligomer out of this list, that targets positions 36-60, is therefore entirely arbitrary and not based on any conscious selection that was made by the applicants when drafting the application as filed.

<u>Claim 1 is an inadmissible intermediate generalisation</u>

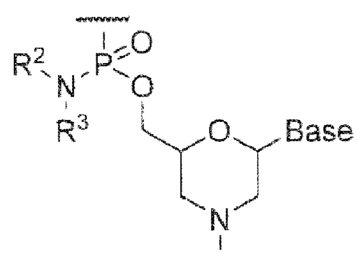
2.5 Turning to our specific objection under this head of attack, it is the opponent's position that the generalisation of the specific example referenced above is an inadmissible intermediate generalisation that is unallowable under Article 123(2) EPC. Specifically, the generalisation of a 2'OMePS RNA antisense oligomer having a sequence that targets positions 36-60 as disclosed in Example 6, out to an antisense oligomer with **<u>any</u>** chemistry, that targets the same region (as recited in claim 1) is unallowable. Based on common general knowledge, and the data presented in the application as filed, the person skilled in the art would be well aware that the chemistry of the oligomer plays a role in determining the skipping efficiency of an antisense oligomer, yet this chemistry is not included in the claim, despite that being the only context in which the oligomer was originally disclosed.

2.6 Oligomers with two different chemistries are tested in the examples: 2'OMePS and phosphorodiamidate morpholino oligomers (PMOs). These two oligomers have different backbone structures. As described in paragraph [0052] of the application as filed, a 2'OMePS oligomer backbone contains a constituent unit represented by the general formula (a) below. The backbone of the oligomer is formed of ribose units connected by phosphorothioate bonds, with the hydroxyl group at position 2' of ribose substituted with a methoxy group. 2'OMePS oligomers can be formed of <u>RNA bases</u> but not DNA bases, because DNA does not have a 2' hydroxyl group that can be methylated.

2.7 As outlined in paragraph [0055] of the application as filed, a PMO can have the general formula (b) below, where R2 and R3 can be the same or different and each represents H, an alkyl, a cycloalkyl or an aryl. PMO oligomers include <u>DNA bases</u> attached to a backbone of methylenemorpholine rings linked by phosphorodiamidate groups.



(a)

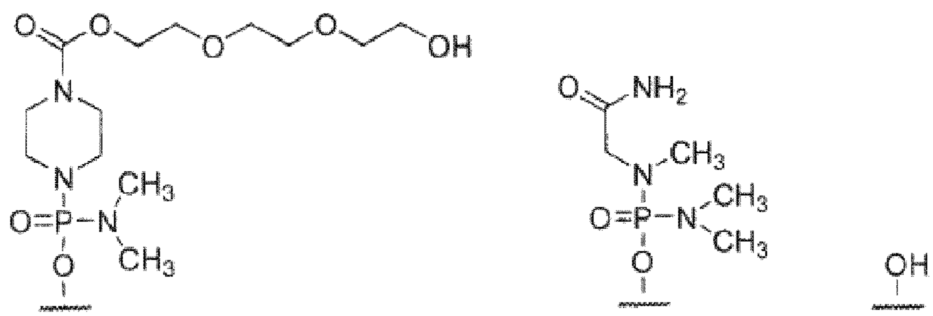


(b)

2.8 These different backbones can affect the skipping efficiencies of antisense oligomers. As stated in D1, which is cited in the background section of the opposed patent at page 1102, second column (emphasis added):

> Initial proof-of-principle clinical trials, using two different AO chemistries (phosphorothioate-linked 2'-*O*-methyl modified bases (2'OMePS) [14] and phosphorodiamidate morpholino oligomer (PMO) [15]) for the targeted skipping of exon 51 of the *DMD* gene after intramuscular injection, have been performed recently with encouraging results. While both chemistries have excellent safety profiles [16,17], <u>PMOs appear to produce more consistent and sustained exon skipping in the *mdx* mouse model of DMD [18–20], in human muscle explants [21], and dystrophic canine muscle cells in vitro [22].</u> However, for some human exons, 2'OMePS and

2.9 In addition, data in the application itself are consistent with this observation, and demonstrate how changes at a single residue of the 5' end of an oligomer can alter their skipping efficiency. As described in paragraph [00110] of the application as filed, the 5' end of the PMO can be one of the following groups:

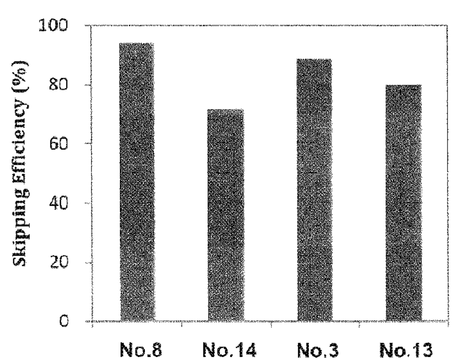


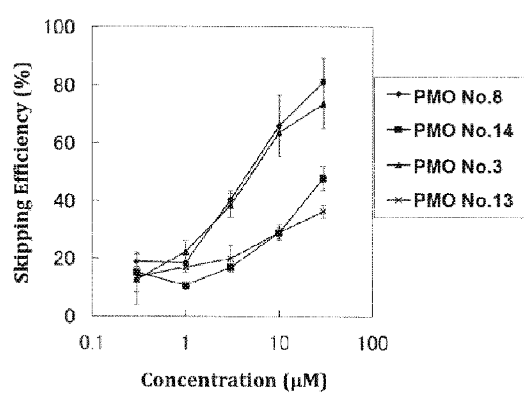
(1) (2) (3)

2.10 Table 2 in the application as filed describes various oligomers that have a variety of different 5' end groups. A summary of PMOs 3, 8, 13 and 14 from this table is provided below. These represent two pairs of PMOs which either have group (1) or group (3) at their 5' end.

PMO No.	Target sequence in exon 53	5' end group	SEQ ID NO:
3	32-56	5' end: group (3)	SEQ ID NO:11
13	32-56	5' end: group (1)	SEQ ID NO:11
8	36-56	5' end: group (3)	SEQ ID NO:35
14	36-56	5' end: group (1)	SEQ ID NO:35

2.11 Figures 8 and 19 of the application (replicated below) show that PMOs 13 and 14 have lower skipping efficiencies than PMO 3 and 8, respectively, which have the same sequence but a different 5' end group. These data demonstrate that changes to one residue at the antisense oligomer 5' end can lead to changes in the skipping efficiency. The skilled person would therefore recognise that the chemical structure of an antisense oligomer is related to the skipping efficiency conferred by that oligomer.





2.12 According to the EPO Guidelines for Examination 2019, H-V-3.2.1:

"*Extracting a specific feature in isolation from an originally disclosed combination of features and using it to delimit claimed subject-matter may be allowed only if there is no structural and functional relationship between the features*" (emphasis added).

As established above, based on data in the application itself and common general knowledge (for example, as evidenced by D1 cited in the background section of the application), the skilled person would be aware that changes in the chemistry of an antisense oligomer can alter its skipping efficiency. A technical relationship therefore exists between the structure of an antisense oligomer and its functional ability to skip exons. As these two features are inextricably linked, extracting one feature – as here, of targeting positions 36-60 of exon 53 – from Example 6 without the 2'OMePS RNA antisense oligomer structure, is an inadmissible intermediate generalisation and so offends against the terms of Article 123(2) EPC.

The application does not provide an overall disclosure justifying the generalising of the antisense oligomer disclosed in Example 6

2.13 Furthermore, the application as filed provides no overall disclosure at all which justifies the generalisation of an antisense oligomer targeting positions 36-60 of exon 53 without also specifying the precise chemical structure of that oligomer. The applicants argued, in their response to the examining division dated 11th March 2019, that the following paragraph provides basis for the general concept of antisense oligomers targeting positions 31-61 of exon 53 with no connection to any particular chemistry (the original translation of this paragraph was corrected during prosecution, but nothing turns on this amendment to the translation).

> [0203] The results are shown in FIGS. 9 to 17. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

2.14 However, this paragraph is pulled out of Example 6 and actually describes conclusions based on experiments conducted with 2'OMePS RNA antisense oligomers. The skilled person would therefore read this paragraph in the specific context of Example 6 and conclude merely that 2'OMePS RNA antisense oligomers targeting positions 31-61 of exon 53 may be useful. There is nothing in this section to warrant any broader interpretation, along the lines espoused by the applicants.

2.15 The applicants then went on to argue that even without this paragraph, the skilled person would recognize, without any doubt, from the application as filed that the specific chemistry utilised in an oligomer was not crucial. However, as explained in detail above, this is contradicted by the application as filed. The skilled person would be fully aware that altering the chemistry of an antisense oligomer is likely to result in changes to its exon skipping efficiency, therefore these conclusions in Example 6 cannot be automatically extrapolated to all antisense oligomers that target the 31-61 region of exon 53.

2.16 Indeed, targeting the 31-61 region of exon 53 is not mentioned anywhere else in the application as filed. In fact, a different region is actually suggested to be the core of the invention in paragraph [0011] of the application as filed:

[0011] As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

2.17 The antisense oligomers that were originally recited in claim 1, and the example nucleotide sequences discussed in paragraphs [0017] and [0030]-[0032] of the application as filed target different sequences to the one now claimed – actually within the region of 31-58, slightly broader than (but tangibly different from) the 32-56 region identified in paragraph [0011]. The inventive concept that was emphasised in the application as filed was to target the region of 31-58, and not the region of 31-61, which is mentioned in Example 6 only. There is, therefore, no overall disclosure at all which justifies the generalisation of this feature.

2.18 In conclusion, the subject matter of claim 1 extends beyond the content of the application as originally filed, because extracting the feature of targeting positions 36-60 of exon 53 from Example 6, without at the same time incorporating the 2'OMePS RNA antisense oligomer structure, is an inadmissible intermediate generalisation. Given that the feature of an antisense oligomer targeting positions 36-60 of exon 53 with any chemistry is an inadmissible intermediate generalisation, dependent claims 2-4 also extend beyond the content of the application as originally filed.

3 LACK OF NOVELTY

3.1 An antisense oligomer that targets positions 36-60 of exon 53, as recited in claim 1, is directly and unambiguously derivable from the disclosure in D2. As shown below, paragraph [0015] of D2 discloses three series of 25 base length molecules denoted by SEQ ID NOs: 10-12 which target three regions of exon 53.

[0015] The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

```
                                        (SEQ ID NO: 10)
j)   CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;
                                        (SEQ ID NO: 11)
k)   CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
or
                                        (SEQ ID NO: 12)
l)   XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,
```

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

3.2 Paragraph [0028] of D2 defines "X" in these sequences as "being thymine (T) or uracil (U)" to account for the fact that the molecule can be, e.g., a PMO molecule (where X = T) or a 2'OMePS molecule (where X = U).

3.3 Paragraph [0030] of D2 defines a molecule in this context as "*any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an* ***oligodeoxyribonucleotide****, an* ***oligoribonucleotide****, a phosphorodiamidate morpholino* ***oligonucleotide*** *(PMO) or a phosphorothioate-linked 2'-O- methyl* ***oligonucleotide*** *(2'OMePS)*" emphasis added. All four of the examples provided in the definition of paragraph [0030] are oligomers.

3.4 The three regions of exon 53 targeted by these antisense oligomers are directly and unambiguously derivable by reverse complementing the sequences of SEQ ID NOs: 10-12. These reverse complemented sequences are then simply aligned with the nucleotide sequence of exon 53 (which was already known at the priority date and is disclosed as SEQ ID NO: 1 in the granted patent) to identify the regions of exon 53 targeted and derive an oligomer of the type claimed.

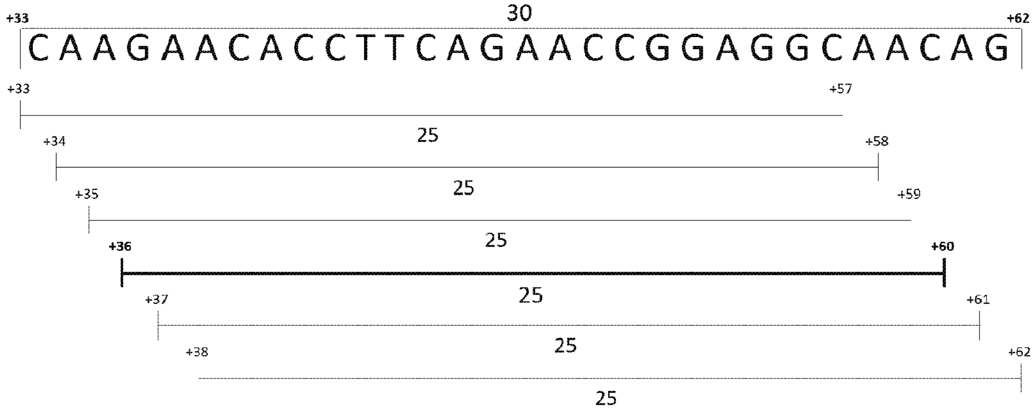
3.5 Furthermore, it is well established in the case law that the technical disclosure in a prior art document must be considered as a whole. Example 2 of D2 discloses various antisense oligomers that target regions of exon 53. PMO-H, PMO-I and PMO-G, which are discussed extensively in Example 2, align with the general formulae provided by SEQ ID NOs: 10-12, as shown in the table below:

Oligomer	Reverse complement sequence (where X = A)	Positions of exon 53 targeted
SEQ ID NO: 10	CXXGXXCXCCTTCXGXXCCGGXGGCXXCXG	positions 33-62
PMO-H	CAAGAACACCTTCAGAACCGGAGGCAACAG	
SEQ ID NO: 11	GXXCXCCTTCXGXXCCGGXGGCXXCXGTTG	positions 36-65
PMO-I	GAACACCTTCAGAACCGGAGGCAACAGTTG	
SEQ ID NO: 12	GTXCXXGXXCACCTTCAGAACCGGAGGCAA	positions 30-59
PMO-G	GTACAAGAACACCTTCAGAACCGGAGGCAA	

3.6 The skilled person, therefore, reading the disclosure of D2 as a whole would directly and unambiguously derive a series of 25 base oligomers that target positions 33-62, 36-65 and 30-59 of exon 53, which would necessarily include positions 36-60 in the derived set. As a further illustration, the step-wise production of these 25 base antisense oligomers across PMO-H (SEQ ID NO: 10) and PMO-I (SEQ ID NO: 11) is demonstrated in the Figure below.

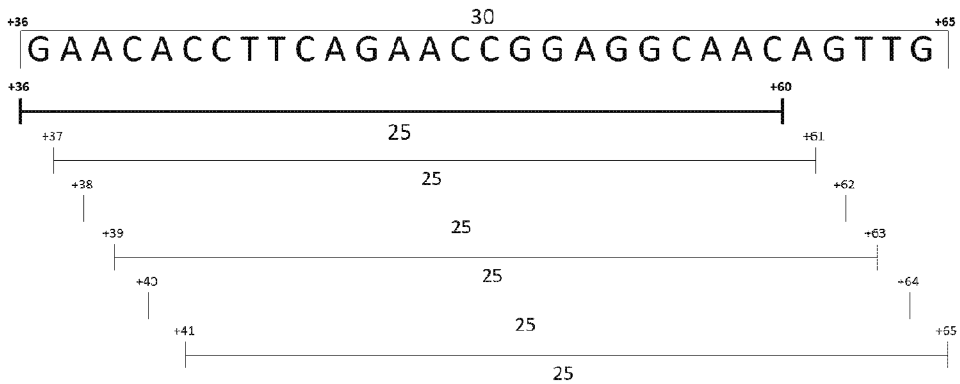
PMO-H (SEQ ID NO: 10)

Targeting positions 33-62 of exon 53



PMO-I (SEQ ID NO: 11)

Targeting positions 36-65 of exon 53



3.7 Figure 1d of D2 shows a similar series of overlapping antisense oligomers that target exon 53. The Figures above clearly demonstrate that the molecules disclosed in paragraph [0015] of D2 include 25 base antisense oligomers targeting positions 36-60 of exon 53, as recited in claim 1. Thus, D2 directly and unambiguously discloses an antisense oligomer that targets positions 36-60 of exon 53, as recited in claim 1, and that subject matter therefore lacks novelty over D2.

3.8 Paragraph [0030] of D2 states that the antisense molecule can be an "*phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS)*". These 2'OMePS RNA antisense oligomers therefore represent an oligonucleotide according to claim 2 which have "*a sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified*" and as required by claim 3 (a methoxy group at the 2' position and the phosphorothioate bonds). Finally, the substitution of the hydroxyl group at position 2' of ribose substituted with a methoxy group falls within the Markush formula that is recited in claim 4. Dependent claims 2-4, therefore also lack novelty in view of D2.

3.9 In conclusion, all of the claims lack novelty according to Article 54 EPC.

4 LACK OF INVENTIVE STEP

4.1 The claims lack an inventive step in view of either D1 or D2. The claimed oligomer represents a routine or arbitrary variant of the prior art antisense oligomers and so fails to meet the requirements of Article 56 EPC.

Claim 1 would have been obvious starting from either D1 or D2

4.2 All of D1, D2 and D4 (Popplewell et al. (2009) *Molecular Therapy*, 3:554-561) are related publications by the Dickson research group based at Royal Holloway University. They detail a series of experiments conducted using PMOs that were designed to target exon 53 of the DMD gene. The data in these three documents overlap to a significant degree; for example, Figures 8-10 and Table 4 of D2 are disclosed in D1 and Figures 1, 3 and 4 of D2 are present in D4.

4.3 In principle, any one of these three documents could be considered to represent the closest prior art in this case, because they are related to the same purpose as the opposed patent, namely the provision of antisense oligomers that target regions of exon 53 of the DMD gene. For the sake of argument, however, D1 has here been taken as the closest prior art, if nothing else because this document was discussed at considerable length during the prosecution of the opposed patent. However, in our view, D2 might be seen to represent an alternative most promising starting point to arrive at the claimed invention, and so some comments have also been provided in this respect.

The antisense oligonucleotides disclosed in D1

4.4 D1 discloses a range of antisense oligomers, but those which are most relevant to granted independent claim 1 are summarised in the table below.

Oligomer name	Position in exon 53	PMO	Oligomer length
PMO-A	35-59	h53A1	25
PMO-G	30-59	h53A30/1	30
PMO-H	33-62	h53A30/2	30

Granted claim 1 targets positions 36-60 of exon 53, which is only one base shifted compared to PMO-A disclosed in D1, as shown in the figure below:

D1 teaches that the skipping activity of an antisense oligomer depends on the model used

4.5 D1 compared the skipping efficiencies of PMOs that target exon 53 in three models: normal human skeletal muscle cells, DMD patient cells and a humanised DMD mouse model. D1 describes how (page 107, column 2, paragraph 2):

> The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent confirmation of optimal sequence(s) for the targeted skipping of exon 53.

While PMO-A, PMO-G and PMO-H demonstrated skipping efficiency in all three models, it was seen that the skipping efficiencies of those oligonucleotides varied depending on the model used.

(a) Human skeletal muscle cells

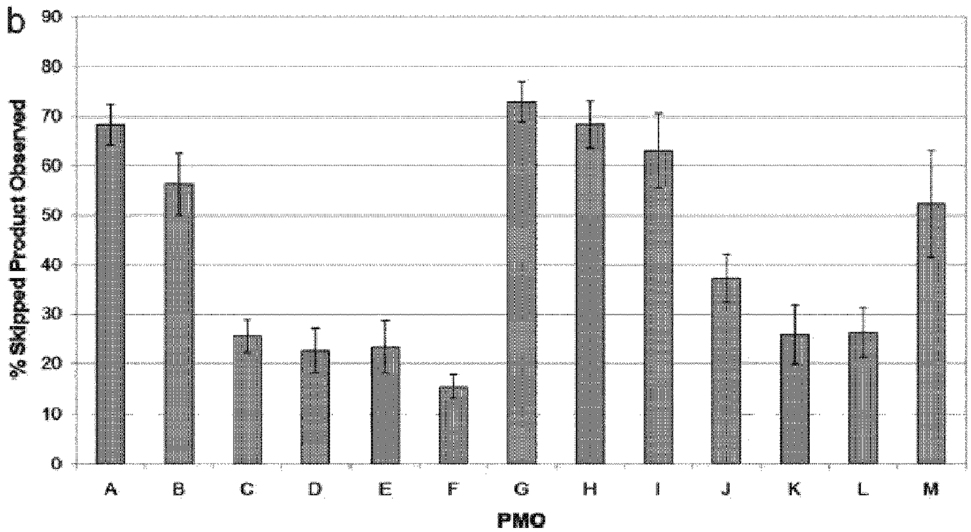
4.6 In normal human skeletal muscle cells, PMO-G and PMO-H were found to have a greater skipping efficiency than PMO-A. The skipping efficiencies of PMO-A, PMO-G and PMO-H were found to be 12.7%, 52.5% and 87.2%, respectively. However, as stated in section 3 on page 104 of D1[1]:

> produced by each in normal hSkMCs. However, studies in normal hSkMCs are limited as they do not allow assessment of the therapeutic effect at the protein level (i.e. dystrophin restoration). Further studies have therefore been performed here to elucidate and confirm which AO(s) would have the potential as a treatment for patients with an eligible deletion. AOs, whose target sites are with-

(b) DMD patient cells

4.7 The bioactivity of PMO-G, PMO-H and PMO-A were directly compared at 300nM by nucleofection in DMD patient cells which had exons 45-52 deleted. As demonstrated in Figure 1b of D1 replicated below, PMO-A, PMO-G and PMO-H have similar exon skipping efficiencies of approximately 70% and were the most active of the PMOs that were tested. Figure 1c of D1 shows that there is no significant difference between the skipping efficiencies of PMO-G and PMO-A, as the p value produced by a two-tailed student's t-test was 0.4496.

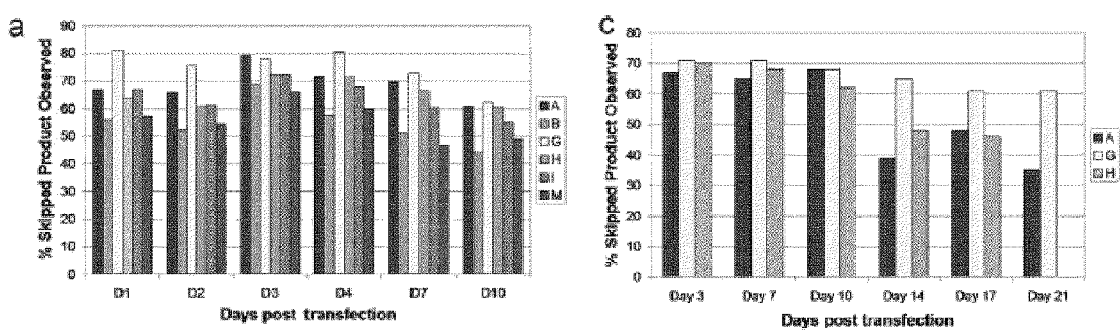
[1] AOs = antisense oligomers



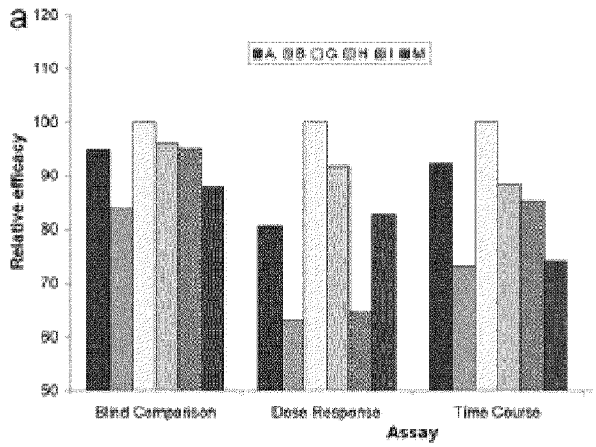
4.8 As detailed in D1 on page 104 at the bottom of column 1, the antisense oligomers tested were divided into groups depending on their skipping efficiency.

> their skipping efficiency. PMOs that produced over 50% exon skipping were designated as Type 1, those that produced between 25% and 50% exon skipping were described as Type 2, while those that produced less than 25% as Type 3. Where appropriate, the

4.9 The activity of the most effective Type 1 PMOs, including PMO-A, PMO-G and PMO-H, were analysed in further experiments. Figures 3a and 3c of D1 replicated below demonstrate that the exon skipping induced by Type 1 PMOs lasted for up to 10 days after transfection. At Day 10 PMO-A, PMO-G and PMO-H retained a 60% exon skipping. A longer time course study showed that PMO-G retained the highest levels of exon skipping, but that PMO-A still retained approximately half of its activity on Day 21.



4.10 As shown in Figure 4a of D1 (replicated below) PMO-A, PMO-G and PMO-H are the three most effective PMOs that were tested across the DMD patient cell assays.



4.11 As outlined in D1 on page 104, column 2, at the bottom of paragraph 3 (emphasis added):

> genase release into culture medium (results not shown). The relative efficacy of the six Type 1 PMOs in the direct comparison, dose–response and time-course assays is summarised in Fig. 4a. Exon 53 skipping by PMO-G is used as the baseline set at 100%. This clearly shows the PMO-G outperforms the other Type 1 PMOs in vitro in patient cells. <u>However, it should also be noted that a number of the other Type 1 PMOs, namely PMO-A and PMO-H, also appear to perform very well across these three comparative tests.</u>

(c) Humanised DMD mouse model

4.12 As discussed in section 3.2 on page 105 of D1, the humanised DMD mouse model is an important model, which allows the *in vivo* action of PMOs to be analysed. In this highly relevant model, PMO-A out-performed both PMO-G and PMO-H.

> Skipping of exon 53 was evident for each of the PMOs tested; average skipping seen in both legs was <u>**8% for PMO-A**</u>, 7.6% for PMO-I, <u>**7.2% for PMO-G**</u>, but a slightly lower level of <u>**4.8% for PMO-H**</u>.

4.13 In conclusion, D1 teaches that the exon skipping efficiency of PMOs is entirely dependent on the model used to conduct the assay. As demonstrated in D1, in human skeletal muscle cells PMO-G and PMO-H were found to have a greater skipping efficiency than PMO-A. However, in the highly relevant humanised DMD mouse model PMO-A had a higher exon skipping efficiency than both PMO-G and PMO-H.

<u>D1 teaches that both 25 and 30 base length antisense oligomers can have high skipping efficiencies</u>

4.14 As set out in detail above, D1 discloses the high skipping efficiency of 25 base length antisense oligomers (PMO-A) and 30 base length antisense oligomers (PMO-G and PMO-H) that target positions 30-62 of exon 53 of the DMD gene. The authors of D1 conclude on page 109, column 2, paragraph 3 that:

> We would therefore recommend that PMOs targeting sequence +30+65 of exon 53 of the *DMD* gene worthy of consideration for any upcoming clinical trial. In this study, sequence +30+65 was effectively targeted by PMOs-A, -B, -G and -H, resulting in exon 53 skipping. Since repeated delivery would be required for therapeutic action, the more persistent action of PMO-G may suggest this to be the PMO of choice for the targeted skipping of exon 53, and PMOs-A and -H providing viable alternatives if required.

4.15 D1 therefore recommends the 30 base antisense oligomer PMO-G as a potential clinical reagent, but the 25 base antisense oligomer PMO-A is also considered to be an entirely worthwhile alternative. However, in the applicants' 1st August 2017 reply to the EPO examining division, it was argued that D1 taught away from 25 base antisense oligomers. As the above discussion should illustrate, that is not in any way an objective and fair view of the disclosure of this document; rather, that position was crafted based on cherry-picking particular data and comments from throughout the document, and it ignores the overall conclusions and more relevant data that are presented when the document is read as a whole.

4.16 On page 2 of that same response, the applicants highlighted the exon skipping activities of PMO-A, -B, -G, -H and -I disclosed in Table 1 on page 105 of D1. Admittedly, these data show that PMO-G has a higher efficiency than PMO-A, but as detailed in section 4.6, the relevance of the data from normal human skeletal muscle cells is rather limited, as this model does not enable the therapeutic effect at the protein level to be assessed. As it happens, the data conducted in DMD patient cells and the humanised DMD mouse model are more relevant in demonstrating the ability of an oligomer to have a therapeutic effect, and these data give different read-outs that do not at all favour the position that the applicants have taken.

4.17 As discussed in detail in sections 4.7 – 4.11 above, PMO-A, PMO-G and PMO-H are the three most effective PMOs that were tested in D1. While PMO-G demonstrates the highest skipping efficiencies, PMO-A also showed very high skipping efficiencies across the various tests performed. In fact, in the highly relevant humanised DMD mouse model PMO-A had a higher exon skipping efficiency than both PMO-G and PMO-H (see section 4.12).

4.18 D1 teaches that there are two factors that have a substantial influence on the skipping efficiencies of an antisense oligomer: (a) the oligomer length and (b) the region of exon 53 targeted. Page 108 of D1 makes the two following points:

> for DMD. The data presented here would indicate that PMOs targeting within the sequence +30+65 of exon 53 (namely PMO-A, -G and -H) produce levels of exon skipping that may be considered effective (over 50% exon skipping). There remains however the

and

> PMO bioactivity. The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. Previous studies by the Leiden

It should be emphasised here that this second statement does not at all mean that 30 base oligomers are always better than 25 base oligomers (as the applicants would have us believe), but instead describes the situation where two oligomers target the same region on exon 53. In

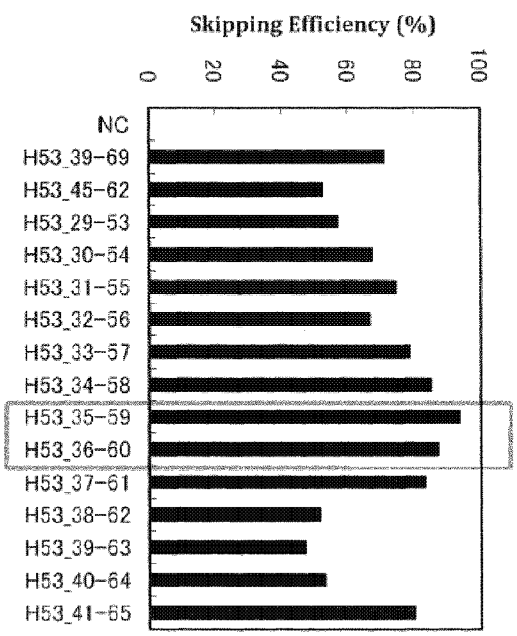
this situation, the 30 base oligomer has been observed to be more bioactive than the 25 base oligomer in cells. However, it is worth reiterating that D1 also reports that PMO-A (a 25 base oligomer) outperformed 30 base oligomers in an animal model.

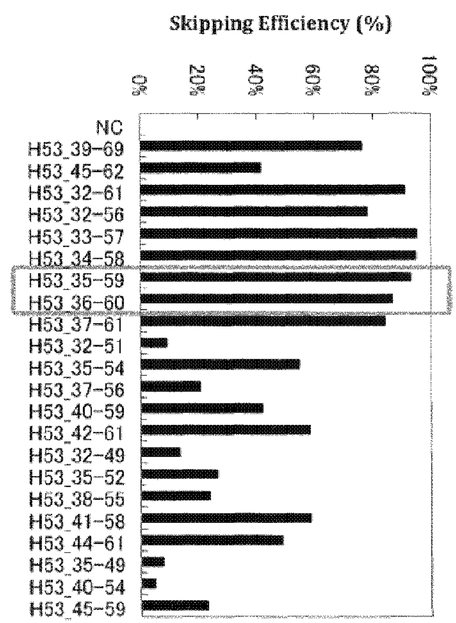
4.19 If anything, these statements therefore teach the person skilled in the art in this instance that the highest activities might be obtained with oligomers that target positions 30-65 of exon 53 **and** are 30 base pairs in length (*i.e.* PMO-G). However, D1 also teaches that shorter oligomers that target positions 30-65 of exon 53, such as PMO-A, can induce effective skipping efficiencies of over 50%, particularly in animals.

Claim 1 would have been obvious starting from D1

4.20 Turning now to the problem-solution approach, D1 can be considered to be the **closest prior art** document, as stated above. D1 discloses a number of antisense oligomers, but the most relevant to this analysis is PMO-A, because it has the highest structural similarity with the claimed antisense oligomer (for example, see T 606/89).

4.21 A **difference** between claim 1 and PMO-A is that the granted antisense oligomer targets positions 36-60 of exon 53, i.e. the target sequence is shifted by one nucleotide base compared to PMO-A. The opposed patent actually includes data that illustrate the technical effect of this difference – as demonstrated in Figures 16 and 17 (replicated below). These figures actually show that an antisense oligomer targeting the same positions as PMO-A (H53_35-59) has a higher skipping efficiency compared to an antisense oligomer targeting the positions of claim 1 (H53_36-60).





4.22 Therefore, the **objective technical problem** to be solved in view of D1 can be seen merely as the provision of an alternative antisense oligomer that targets exon 53. The oligomer claimed targets positions 36-60 of exon 53 – only one base different to the region targeted by PMO-A! This would have required no application of inventive skill, and is *prima facie* **entirely obvious**. On top of that, these data highlighted above illustrate that even in the assay chosen by the

applicants, the claimed antisense oligomer is objectively worse than the nearest structural homologue in the closest prior art.

4.23 This is even more the case given that the closest prior art D1 specifically suggests antisense oligomers targeting the general region of exon 53 targeted by PMO-A and the claimed oligomer, namely positions 30-65 (see section 4.14 above for details). The skilled person would therefore have had a high expectation of success that modifying the region targeted by the antisense oligomer by just one base, within a region that had already been highlighted as relevant for successful exon skipping, would result in an antisense oligomer that could effectively function to skip exon 53.

4.24 For the sake of completeness, consider the following. Even if, following the logic espoused by the applicants during examination, the person skilled in the art had instead actually commenced its research starting from the antisense oligomer PMO-G disclosed in D1, perhaps because it had the highest skipping efficiency in DMD patient cells, they would have still arrived at claim 1 without using inventive skill. The skilled person would be well aware from D1 itself that 25 base antisense oligomers, whilst sometimes being less bioactive than their 30 base counterparts, can still produce very effective exon skipping efficiencies. So if the skilled person had combined the disclosures of PMO-G, PMO-H and PMO-A in D1, they would have had an entirely reasonable expectation that choosing a 25 base region that is nearly entirely encompassed within the region targeted by PMO-G, only one base away from PMO-A, and which falls entirely within the region targeted by PMO-H would successfully produce an effective antisense oligomer.

4.25 Additional prior art, for example D2, corroborate that it was an entirely reasonable expectation that choosing a 25 base region would successfully produce an effective antisense oligomer. As stated above, D2 is a related publication by same research group as D1. That document demonstrates that 25 base length antisense oligomers targeting exon 53 are entirely effective. For example, claim 1 of D2 recites:

"*An oligomer for ameliorating DMD, the oligomer comprising* ***at least 25 contiguous bases*** *of a base sequence selected from the group consisting of…*" (emphasis added).

4.26 D3 (WO 2010/048586) also teaches the skilled person that 25 base pair antisense oligomers targeting exon 53 can effectively induce exon skipping. Example 3 of D3 (the paragraph spanning pages 75-76) describes three different 25 base oligomers targeting exon 53 that produced effective exon skipping. Therefore, if the skilled person were to combine D1 with either D2 or D3, this would further confirm that 25 base oligomers were entirely capable of inducing effective skipping of exon 53.

4.27 In summary, from the teachings in D1 alone, or in combination with either D2 or D3, the claimed antisense oligomer is an obvious alternative. The skilled person would have been able to predict with a high degree of certainty that an antisense oligomer which is only one base different from the effective antisense oligomer PMO-A disclosed in D1 would be able to effectively induce exon skipping. This is all the more the case given that the claimed oligomer falls within the broad region of 30-65 of exon 53, which D1 identified as being important in order to induce effective exon skipping.

4.28 For completeness, these arguments apply *mutatis mutandis* starting from D2 as the closest prior art, because D1 and D2 are related publications by the same research group published at a similar time and include very similar data and conclusions.

4.29 Dependent claims 2-4 are also obvious in view D1 or D2, either taken alone or in combination, as they recite known structural modifications that can be made to an antisense oligomers. For example, D1 discloses antisense oligomers having a 2'OMePS chemistry (see page 1102, second column in section 2.8 above). As detailed in section 2.6, 2'OMePS are antisense oligomers formed of ribose units connected by phosphorothioate bonds, with the hydroxyl group at position 2' of ribose substituted with a methoxy group. These 2'OMePS RNA antisense oligomers therefore represent an oligonucleotide according to claim 2 which have "*a sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified*" as required by claim 3 (a methoxy group at the 2' position and the phosphorothioate bonds). Finally, the substitution of the hydroxyl group at position 2' of ribose substituted with a methoxy group falls within the Markush formula that is recited in claim 4.

4.30 In conclusion, all of the claims lack an inventive step according to Article 56 EPC.

5 INSUFFICIENT DISCLOSURE

5.1 The patent does not describe the invention in a sufficiently clear and complete manner for the skilled person to put the invention into practice across the entire claim scope without undue burden and inventive skill. Accordingly, the requirements of Article 83 EPC are not satisfied.

5.2 The patent provides data showing the exon skipping efficiency of a <u>2'OMePS</u> RNA antisense oligomer targeting positions 36-60 of exon 53 in a human rhabdomyosarcoma cell line. However, the antisense oligomer recited in claim 1 is not limited to any particular chemistry, neither does it define the system in which the skipping efficiency is assayed. No data at all are provided demonstrating that antisense oligomers with different chemistries targeting positions 36-60 might have effective exon skipping efficiencies, or that 2'OMePS RNA antisense oligomers have similar efficiencies in any other model systems, such as in DMD patient cells or in a humanised DMD mouse model.

5.3 Indeed, given the data in the patent, there are doubts whether antisense oligomers with <u>any</u> particular chemistry, that target positions 36-60 of exon 53, might share an effective exon skipping efficiency. As discussed in detail in sections 2.8-2.11 above, the patent shows that minor changes in the backbone chemistry of an antisense oligomer can result in changes to the exon skipping efficiencies observed. In particular, Figure 19 of the patent (replicated in section 2.11 above) demonstrates that a simple change at the 5' end of the molecule can change antisense oligomers with an exon skipping frequencies of approximately 80% (PMOs No.3 and No.8) into less effective antisense oligomers with an exon skipping efficiency of under 50% (PMOs No.13 and No.14). Therefore, the patent itself includes proof that the chemistry of the antisense oligomer plays a role in the exon skipping efficiency observed.

5.4 In the applicants' reply to the search report dated 10th November 2016, an indirect comparison was made between the claimed antisense oligomer and PMO-G disclosed in D1. However, this comparison involved antisense oligomers with two different chemistries – PMOs and 2'OMePS oligomers. The applicants highlighted that PMO-G (the oligomer disclosed in D1) was disclosed in the application as PMO No.12 and No.15 with SEQ ID NO:39 and stated that:

> In Figures 2 and 3 of the present application it is shown that SEQ ID NO:11 corresponding to H53_32-56 (SEQ ID NO:11) corresponding to PMO No. 3 has a higher activity than the oligomer disclosed by Popplewell et al.
>
> Furthermore, H53_36-60 (SEQ ID NO:57) has a higher skipping activity than H53_32-56 (SEQ ID NO:11) as shown in Figures 16 and 17.
>
> From the above explanations it is clear that H53_36-60 (SEQ ID NO:57) has higher skipping activities than the best oligomer disclosed by Popplewell et al. This could not be expected from

These comparisons are inappropriate. Figures 2 and 3 are based on data conducted with PMOs in human DMD patient-derived fibroblasts (see Example 2), whereas Figures 17 and 18 are based on data testing the activity of 2'OMePS oligomers in human rhabdomyosarcoma cells (Example 6). As discussed in detail above, the system in which an oligomer is tested and the chemistry of the oligomer affect the exon skipping efficiency observed. Therefore, an indirect comparison cannot at all be validly made between the two data sets.

5.5 Figures 2-3, referenced by the applicants, show that PMO No. 12, which targets the same region of exon 53 as PMO-G in D1, has a lower exon skipping efficiency than a PMO targeting the positions 32-56 of exon 53. In fact, the skipping efficiencies observed in the patent for PMO No. 12 in TIG-119 cells (human normal tissue-derived fibroblasts) and 5017 cells (human DMD patient-derived fibroblasts) were less than 5%. This is significantly different to the exon skipping efficiency observed with PMO-G in D1, where a 52.5% and approximately 70% skipping efficiency was observed in normal human skeletal cells and DMD patient cells, respectively. In the patent, the 5' end of PMO No. 12 was modified to group (2), whereas PMO-G in D1 does not possess a 5' modification. Therefore, it is evident that the systems used to test the exon skipping and the oligomer chemistries differ completely between D1 and Example 2, which may explain the differences between the skipping efficiencies observed.

5.6 In the same vein, Figure 17 of the patent shows that a 2'OMePS antisense oligomer targeting the same region of exon 53 as PMO-A (in D1) is actually more effective than 2'OMePS antisense oligomers that target the claimed region of 36-60 and positions 32-56. If an indirect comparison between Figures 2-3 and Figure 7 is made, the data actually show that PMO-A is <u>more</u> effective than PMO-G, which contradicts the *in vitro* cell data presented in D1. It is therefore evident that the system in which an oligomer is tested and the chemistry of the oligomer are highly relevant to the skipping efficiency observed. Therefore indirect comparisons between data presented in Examples 2 and 6 cannot be made.

5.7 The contradictions in the indirect comparisons made with D1 and the data presented in Figure 19 therefore show that there are doubts whether antisense oligomers with any particular chemistry targeting positions 36-60 of exon 53 would produce effective skipping efficiencies. Furthermore, the level of exon skipping observed is also dependent on the system in which the oligomer is tested. The patent therefore does not describe the broad class of antisense oligomers that are claimed, which target positions 36-60 of exon 53, in a sufficiently clear and complete manner for the skilled person to put the invention into practice without undue burden. The patent is therefore insufficient according to Article 83 EPC.

6 CITED DOCUMENTS

Documents already cited during search/examination

D1: Popplewell *et al.* (2010) *Neuromuscular Disorders*, 20:102-110.

D2: US 2010/0168212

D3: WO 2010/048586

New cited documents

D4: Popplewell *et al.* (2009) *Molecular Therapy*, 3:554-561.

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Munich, January 21, 2022

Our Ref.: 223 974 m2/sr
European Patent 3 018 211
NIPPON SHINYAKU CO., LTD. et al.
Opposition Proceedings

The Patent Proprietor no longer approves of the text in which the above-mentioned patent was granted and will not submit an amended text. Our request for oral proceedings is withdrawn.

JOSEPH P. TAORMINO, PH.D.
European Patent Attorney
Hoffmann Eitle Patent- und Rechtsanwälte PartmbB
Association No. 151

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Date	
	31.01.2022

Reference	Application No./Patent No.
223974	15199455.5 - 1111 / 3018211
Applicant/Proprietor	
Nippon Shinyaku Co., Ltd., et al	

Decision revoking the European Patent (Art. 101(3)(b) EPC)

European Patent No. 3018211 is revoked.

Reasons for the decision:
The patent proprietor, by statement filed on 21.01.2022

☐ requested that the patent be revoked.

☑ stated that he no longer approves the text in which the patent was granted.

As a consequence there is no longer a version of the text submitted and/or approved by the patent proprietor (Art. 113(2) EPC), in which the patent can be maintained, therefore the European Patent must be revoked pursuant to Article 101(3)(b) EPC.

Means of redress

Appeal
This decision is open to appeal. Attention is drawn to the attached text of Articles 106 to 108 EPC and Rules 97 and 98 EPC.

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Enclosure: EPO Form 2019
EPO Form 2310A

Registered Letter
EPO Form 2345 07.19 (24/01/22) REVO/MESY

to EPO postal service: 24.01.22

Article 106
Decisions subject to appeal

(1) An appeal shall lie from decisions of the Receiving Section, Examining Divisions, Opposition Divisions and the Legal Division. It shall have suspensive effect.

(2) A decision which does not terminate proceedings as regards one of the parties can only be appealed together with the final decision, unless the decision allows a separate appeal.

(3) The right to file an appeal against decisions relating to the apportionment or fixing of costs in opposition proceedings may be restricted in the Implementing Regulations.

Rule 97
Appeal against apportionment and fixing of costs

(1) The apportionment of costs of opposition proceedings cannot be the sole subject of an appeal.

(2) A decision fixing the amount of costs of opposition proceedings cannot be appealed unless the amount exceeds that of the fee for appeal.

Rule 98
Surrender or lapse of the patent

The decision of an Opposition Division may be appealed even if the European patent has been surrendered in all the designated Contracting States or has lapsed in al those States.

Article 107
Persons entitled to appeal and to be parties to appeal proceedings

Any party to proceedings adversely affected by a decision may appeal. Any other parties to the proceedings shall be parties to the appeal proceedings as of right.

Article 108
Time limit and form

Notice of appeal shall be filed in accordance with the Implementing Regulations, at the European Patent Office within **two months** of notification of the decision. Notice of appeal shall not be deemed to have been filed until the fee for appeal has been paid. Within **four months** of notification of the decision, a statement setting out the grounds of appeal shall be filed in accordance with the Implementing Regulations.

Further information concerning the filing of an appeal

(a) Notice of appeal can be filed in accordance with Rule 1 and Rule 2(1) EPC, by delivery by hand, by post, or by technical means of communication. The filing has to comply with the details and conditions and, where appropriate, any special formal or technical requirements laid down by the President of the European Patent Office (R. 99(3) EPC).

(b) The addresses of the filing offices of the European Patent Office are as follows:

(i) European Patent Office
D-80298 Munich
Germany
Fax : +49 89 2399-4465

(ii) European Patent Office
Postbus 5818
NL-2280 HV Rijswijk (ZH)
The Netherlands
Fax : +31 70 340-3016

(iii) European Patent Office
D-10958 Munich
Germany
Fax : +49 30 259 01-840

(c) The notice of appeal must contain the name and address of the appellant in accordance with the provisions of Rule 41(2)(c) EPC, an indication of the decision impugned, and a request defining the subject of the appeal. In the statement of grounds of appeal the appellant shall indicate the reasons for setting aside the decision impugned, or the extent to which it is to be amended, and the facts and evidence on which the appeal is based (R. 99(1) and (2) EPC). The notice of appeal and any subsequent submissions stating the grounds for appeal must be signed (R. 50(3) EPC).

(d) The fee for appeal is laid down in the Rules relating to Fees. The schedule of fees and expenses of the EPO or a reference to the current version is regularly published in the Official Journal of the European Patent Office under the heading "Guidance for the payment of fees, expenses and prices". Fee information is also published on the EPO website under www.epo.org/ fees.

EXHIBIT AQ

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A61K	48/00	(2006.01)	A61K	48/00	
A61K	31/7088	(2006.01)	A61K	31/7088	
A61K	31/712	(2006.01)	A61K	31/712	

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(54)【発明の名称】アンチセンス核酸

(57)【特許請求の範囲】
【請求項１】
ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマーであって、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第36～60番目のヌクレオチドからなる配列に相補的な塩基配列からなる、アンチセンスオリゴマー。
【請求項２】
オリゴヌクレオチドである、請求項1に記載のアンチセンスオリゴマー。
【請求項３】
前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドの糖部分及び/又はリン酸結合部分が修飾されている、請求項2に記載のアンチセンスオリゴマー。
【請求項４】
前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドの糖部分が、2’位の-OH基が、OR、R、R’OR、SH、SR、NH_2、NHR、NR_2、N_3、CN、F、Cl、Br及びIからなる群より選択されるいずれかの基で置換されたリボースである、請求項3に記載のアンチセンスオリゴマー。
（上記Rは、アルキル又はアリールを示し、上記R’は、アルキレンを示す。）
【請求項５】
前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドのリン酸結合部分が、ホスホロチオエート結合、ホスホロジチオエート結合、アルキルホスホネート結合、ホ

スホロアミデート結合、及びボラノフォスフェート結合からなる群より選択されるいずれか1つのものである、請求項3又は4に記載のアンチセンスオリゴマー。

【請求項６】

モルホリノオリゴマーである、請求項1に記載のアンチセンスオリゴマー。

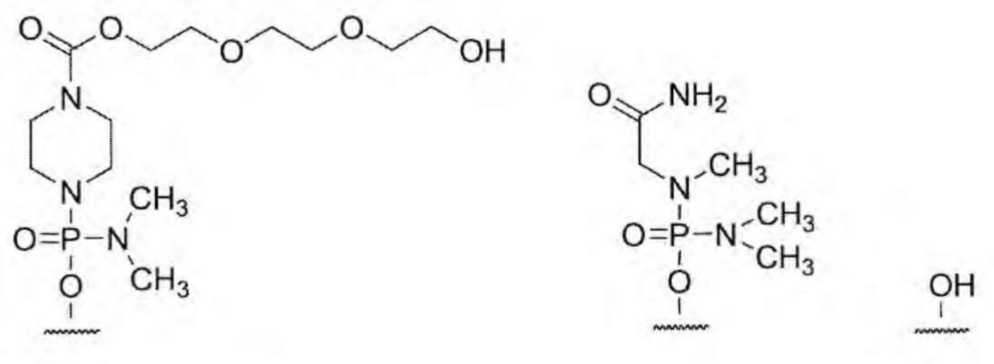
【請求項７】

ホスホロジアミデートモルホリノオリゴマーである、請求項6に記載のアンチセンスオリゴマー。

【請求項８】

5'末端が、下記化学式（1）～（3）のいずれかの基である、請求項6又は7に記載のアンチセンスオリゴマー。

【化２４】



（1） （2） （3）

【請求項９】

配列番号57に示す塩基配列からなる、請求項1～8のいずれか1項に記載のアンチセンスオリゴマー。

【請求項１０】

請求項1～9のいずれか1項に記載のアンチセンスオリゴマー、その医薬的に許容可能な塩又は水和物を有効成分とする、筋ジストロフィー治療用医薬組成物。

【発明の詳細な説明】

【技術分野】

【０００１】

本発明は、ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマー及び該オリゴマーを含む医薬組成物に関する。

【背景技術】

【０００２】

デュシェンヌ型筋ジストロフィー（DMD）は出生男子約3,500人に1人が発症する最も頻度の高い遺伝性進行性筋萎縮症である。乳幼児期には正常のヒトとほとんど変わらない運動機能を示すが、4～5歳頃から筋力低下がみられる。その後筋力低下は進行し12歳頃までに歩行不能になり、20歳代で心不全または呼吸器不全により死に至る重篤な疾患である。現在、DMDに対する有効な治療法はなく、新たな治療薬の開発が強く求められている。

【０００３】

DMDはジストロフィン遺伝子の変異が原因であることが知られている。ジストロフィン遺伝子はX染色体に存在し、220万塩基対のDNAから成る巨大な遺伝子である。DNAからmRNA前駆体に転写され、さらにスプライシングによりイントロンが除かれ79のエクソンが結合したmRNAが合成される。このmRNAから3,685のアミノ酸に翻訳され、ジストロフィンタンパク質が生成される。ジストロフィンタンパク質は筋細胞の膜安定性の維持に関与しており、筋細胞を壊れにくくするために必要である。DMD患者のジストロフィン遺伝子は変異を有するため、筋細胞において機能を有するジストロフィンタンパク質が殆ど発現されな

い。そのため、DMD患者体内では、筋細胞の構造を維持できなくなり、多量のカルシウムイオンが筋細胞内に流れ込む。その結果、炎症に似た反応が生じ、線維化が進むために筋細胞が再生されにくくなる。

【０００４】

ベッカー型筋ジストロフィー（BMD）もジストロフィン遺伝子の変異が原因であるが、その症状は筋萎縮による筋力低下を呈するものの一般にDMDと比較して軽く、筋力低下の進行も遅く、多くの場合、成人期に発症する。DMDとBMDとの臨床症状の違いは、変異によりジストロフィンのmRNAがジストロフィンタンパク質へと翻訳される際のアミノ酸読み取り枠が破壊されるか、あるいは維持されるかによるものと考えられている（非特許文献1）。つまり、DMDでは、アミノ酸読み取り枠がずれる変異を有することにより、機能を持つジストロフィンタンパク質がほとんど発現しないが、BMDでは変異によりエクソンの一部は欠失しているが、アミノ酸読み取り枠は維持されているために不完全ながらも機能を有するジストロフィンタンパク質が産生される。

【０００５】

DMDの治療法として、エクソンスキッピング法が期待されている。この方法は、スプライシングを改変することでジストロフィンのmRNAのアミノ酸読み取り枠を修復し、部分的に機能を回復したジストロフィンタンパク質の発現を誘導する方法である（非特許文献2）。エクソンスキッピングの対象となるアミノ酸配列部分は失われることになる。そのためこの治療で発現されるジストロフィンタンパク質は正常のものより短くなるが、アミノ酸読み取り枠が維持されるために筋細胞を安定化する機能が部分的に保持される。従って、エクソンスキッピングにより、DMDは、より軽症のBMDと同じような症状を呈するようになると期待されている。エクソンスキッピング法は、マウスやイヌによる動物実験を経て、ヒトDMD患者に対する臨床試験が行われている。

【０００６】

エクソンスキッピングは、5’若しくは3’スプライス部位のいずれか若しくは両方、又はエクソンの内部を標的とするアンチセンス核酸の結合により誘導することができる。エクソンは両方のスプライス部位がスプライソソーム複合体によって認識された場合のみmRNAに包含される。従って、スプライス部位をアンチセンス核酸でターゲッティングすることにより、エクソンスキッピングを誘導することができる。また、エクソンがスプライシングの機構に認識されるためにはエクソンスプライシングエンハンサー（ESE）へのSRタンパク質の結合が必要であると考えられており、ESEをターゲッティングすることでもエクソンのスキッピングを誘導することができる。

【０００７】

ジストロフィン遺伝子の変異はDMD患者によって異なるため、遺伝子変異の場所や種類に応じたアンチセンス核酸が必要になる。これまでに、西オーストラリア大学のSteve Wiltonらによって79個全てのエクソンに対してエクソンスキッピングを誘導するアンチセンス核酸が作製されており（非特許文献3）、オランダのAnnemieke Aartsma-Rusらによって39種類のエクソンに対してエクソンスキッピングを誘導するアンチセンス核酸が作られている（非特許文献4）。

【０００８】

全DMD患者の8%程度は、第53番目のエクソン（以下、「エクソン53」という）をスキッピングすることで治療可能と考えられている。近年では、ジストロフィン遺伝子のエクソン53をエクソンスキッピングのターゲットとした研究について、複数の研究機関から報告がなされている（特許文献1～4；非特許文献5）。しかしながら、エクソン53を高効率にスキッピングさせる技術は、いまだに確立されていない。

【先行技術文献】

【特許文献】

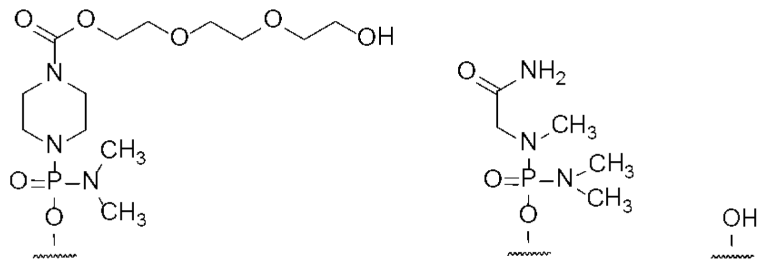
【０００９】

【特許文献１】：　国際公開公報 WO 2006/000057

【特許文献２】：　国際公開公報 WO 2004/048570

【特許文献３】： 米国特許公開公報 US 2010/0168212
【特許文献４】： 国際公開公報 WO 2010/048586
【非特許文献】
【００１０】
【非特許文献１】： Monaco A. P. et al., Genomics 1988; 2: p. 90-95
【非特許文献２】： Matsuo M., Brain Dev 1996; 18: p. 167-172
【非特許文献３】： Wilton S. D., e t al., Molecular Therapy 2007: 15: p. 1288-96
【非特許文献４】： Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77
【非特許文献５】： Linda J. Popplewell et al., (2010) Neuromuscular Disorders , vol. 20, no. 2, p. 102-10
【発明の開示】
【００１１】
上記のような状況において、ジストロフィン遺伝子のエクソン53のスキッピングを強く誘導するアンチセンスオリゴマー及びそのオリゴマーを含む筋ジストロフィー治療薬が望まれている。
本発明者らは、ジストロフィン遺伝子の構造を詳細に研究した結果、ジストロフィン遺伝子のmRNA前駆体（以下、「pre-mRNA」という）のうちエクソン53の5’末端から第32～56番目周辺のヌクレオチドからなる配列をアンチセンスオリゴマーでターゲッティングすることにより、高効率にエクソン53のスキッピングを誘導できることを見出した。本発明者らは、この知見に基づき、本発明を完成させた。
【００１２】
即ち、本発明は、以下のとおりである。
[1] ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマーであって、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第31～53番目、第31～54番目、第31～55番目、第31～56番目、第31～57番目、第31～58番目、第32～53番目、第32～54番目、第32～55番目、第32～56番目、第32～57番目、第32～58番目、第33～53番目、第33～54番目、第33～55番目、第33～56番目、第33～57番目、第33～58番目、第34～53番目、第34～54番目、第34～55番目、第34～56番目、第34～57番目、第34～58番目、第35～53番目、第35～54番目、第35～55番目、第35～56番目、第35～57番目、第35～58番目、第36～53番目、第36～54番目、第36～55番目、第36～56番目、第36～57番目又は第36～58番目のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列からなる、アンチセンスオリゴマー。
[2] オリゴヌクレオチドである、前記[1]に記載のアンチセンスオリゴマー。
[3] 前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドの糖部分及び/又はリン酸結合部分が修飾されている、前記[2]に記載のアンチセンスオリゴマー。
[4] 前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドの糖部分が、2’位の-OH基が、OR、R、R’OR、SH、SR、NH_2、NHR、NR_2、N_3、CN、F、Cl、Br及びIからなる群より選択されるいずれかの基で置換されたリボースである、前記[3]に記載のアンチセンスオリゴマー。
（上記Rは、アルキル又はアリールを示し、上記R’は、アルキレンを示す。）
[5] 前記オリゴヌクレオチドを構成する少なくとも1つのヌクレオチドのリン酸結合部分が、ホスホロチオエート結合、ホスホロジチオエート結合、アルキルホスホネート結合、ホスホロアミデート結合、及びボラノフォスフェート結合のからなる群より選択されるいずれか1つのものである、前記[3]又は[4]に記載のアンチセンスオリゴマー。
[6] モルホリノオリゴマーである、前記[1]に記載のアンチセンスオリゴマー。
[7] ホスホロジアミデートモルホリノオリゴマーである、前記[6]に記載のアンチセンスオリゴマー。
[8] 5’末端が、下記化学式（1）～（3）のいずれかの基である、前記[1]～[7]のいずれ

か1項に記載のアンチセンスオリゴマー。
【化１】



（１）　（２）　（３）

[9]　ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第32～56番目又は第36～56番目のヌクレオチドからなる配列に相補的な塩基配列からなる、前記[1]～[8]のいずれか1項に記載のアンチセンスオリゴマー。
[10]　配列番号2～37からなる群より選択されるいずれか1つの塩基配列からなる、前記[1]～[8]のいずれか1項に記載のアンチセンスオリゴマー。
[11]　配列番号11、17、23、29及び35からなる群より選択されるいずれか1つの塩基配列からなる、前記[1]～[8]のいずれか1項に記載のアンチセンスオリゴマー。
[12]　配列番号11又は35のいずれか1つの塩基配列からなる、前記[1]～[8]のいずれか1項に記載のアンチセンスオリゴマー。
[13]　前記[1]～[12]のいずれか1項に記載のアンチセンスオリゴマー、その医薬的に許容可能な塩又は水和物を有効成分とする、筋ジストロフィー治療用医薬組成物。
【００１３】
本発明のアンチセンスオリゴマーにより、ヒトジストロフィン遺伝子のエクソン53のスキッピングを高効率に誘導することが可能である。また、本発明の医薬組成物を投与することにより、デュシェンヌ型筋ジストロフィーの症状を、効果的に軽減することができる。
【図面の簡単な説明】
【００１４】
【図１】ヒト横紋筋肉腫細胞株（RD細胞）におけるヒトジストロフィン遺伝子エクソン53のスキッピング効率を示す図である。
【図２】ヒト正常組織由来線維芽細胞（TIG-119細胞）にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図３】ヒトDMD患者由来線維芽細胞（5017細胞）にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図４】ヒトDMD患者（エクソン45-52欠失）由来線維芽細胞にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図５】ヒトDMD患者（エクソン48-52欠失）由来線維芽細胞にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図６】ヒトDMD患者（エクソン48-52欠失）由来線維芽細胞にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図７】ヒトDMD患者（エクソン45-52欠失またはエクソン48-52欠失）由来線維芽細胞にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。

【図８】ヒトDMD患者（エクソン45-52欠失）由来線維芽細胞にヒトmyoD遺伝子を導入して筋肉細胞に分化誘導した細胞におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率を示す図である。
【図９】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１０】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１１】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１２】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１３】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１４】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１５】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１６】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１７】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のスキッピング効率（2’-OMe-S-RNA）を示す図である。
【図１８】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のオリゴマー濃度別のスキッピング効率を示す図である。
【図１９】ヒト横紋筋肉腫細胞（RD細胞）におけるヒトジストロフィン遺伝子のエクソン53のオリゴマー濃度別のスキッピング効率を示す図である。

【発明を実施するための最良の形態】

【００１５】

以下、本発明を詳細に説明する。以下の実施の形態は、本発明を説明するための例示であり、本発明をこの実施の形態のみに限定する趣旨ではない。本発明は、その要旨を逸脱しない限り、様々な形態で実施をすることができる。

なお、本明細書において引用した全ての文献、および公開公報、特許公報その他の特許文献は、参照として本明細書に組み込むものとする。また、本明細書は、2010年9月1日に出願された本願優先権主張の基礎となる日本国特許出願（特願2010-196032号）の明細書及び図面に記載の内容を包含する。

【００１６】

1. アンチセンスオリゴマー

本発明は、ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマーであって、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第31～53番目、第31～54番目、第31～55番目、第31～56番目、第31～57番目、第31～58番目、第32～53番目、第32～54番目、第32～55番目、第32～56番目、第32～57番目、第32～58番目、第33～53番目、第33～54番目、第33～55番目、第33～56番目、第33～57番目、第33～58番目、第34～53番目、第34～54番目、第34～55番目、第34～56番目、第34～57番目、第34～58番目、第35～53番目、第35～54番目、第35～55番目、第35～56番目、第35～57番目、第35～58番目、第36～53番目、第36～54番目、第36～55番目、第36～56番目、第36～57番目又は第36～58番目のヌクレオチドからなる配列（以下、「標的配列」ともいう。）のいずれか1つに相補的な塩基配列からなる、アンチセンスオリゴマー（以下、「本発明のオリゴマー」という）を提供する。

【００１７】

［ヒトジストロフィン遺伝子の第53番目のエクソン］

本発明において、「遺伝子」には、ゲノム遺伝子以外に、cDNA、mRNA前駆体及びmRNAも

含まれる。好ましくは、遺伝子は、mRNA前駆体、即ち、pre-mRNAである。

ヒトゲノムにおいて、ヒトジストロフィン遺伝子は遺伝子座Xp21.2に存在する。ヒトジストロフィン遺伝子は、3.0 Mbpのサイズを有しており、既知のヒト遺伝子としては最大の遺伝子である。但し、ヒトジストロフィン遺伝子のコード領域はわずか14kbに過ぎず、該コード領域は79個のエクソンとしてジストロフィン遺伝子内に分散している（Roberts, RG., et al., Genomics, 16: 536-538 (1993)）。ヒトジストロフィン遺伝子の転写物であるpre-mRNAは、スプライシングを受けて14kbの成熟mRNAを生成する。ヒトの野生型ジストロフィン遺伝子の塩基配列は公知である（GenBank Accession No. NM_004006）。

ヒトの野生型ジストロフィン遺伝子のエクソン53の塩基配列を配列番号1に示す。

【００１８】

本発明のオリゴマーは、ヒトジストロフィン遺伝子のエクソン53のスキッピングにより、DMD型ジストロフィン遺伝子でコードされるタンパク質を、BMD型ジストロフィンタンパク質に改変することを目的として作製されたものである。従って、本発明のオリゴマーのエクソンスキッピングの対象となるジストロフィン遺伝子のエクソン53には、野生型だけではなく、変異型も含まれる。

変異型のヒトジストロフィン遺伝子のエクソン53は、具体的には、以下の（a）又は（b）に記載のポリヌクレオチドである。

（a）配列番号1の塩基配列と相補的な塩基配列からなるポリヌクレオチドとストリンジェントな条件下でハイブリダイズするポリヌクレオチド；

（b）配列番号1の塩基配列に対して、90％以上の同一性を有する塩基配列からなるポリヌクレオチド

【００１９】

本明細書中、「ポリヌクレオチド」とは、DNA又はRNAを意味する。

本明細書中、「ストリンジェントな条件下でハイブリダイズするポリヌクレオチド」とは、例えば、配列番号1の塩基配列と相補的な塩基配列からなるポリヌクレオチドの全部又は一部をプローブとして、コロニーハイブリダイゼーション法、プラークハイブリダイゼーション法又はサザンハイブリダイゼーション法などを用いることにより得られるポリヌクレオチドをいう。ハイブリダイゼーションの方法としては、例えば、"Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001"及び"Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997"などに記載されている方法を利用することができる。

【００２０】

本明細書中、「相補的な塩基配列」とは、対象となる塩基配列とワトソン・クリック対を形成する塩基配列に限定されるものではなく、揺らぎ塩基対（Wobble base pair）を形成する塩基配列も含む。ここで、ワトソン・クリック対とは、アデニン-チミン、アデニン-ウラシル及びグアニン-シトシン間に水素結合が形成される塩基対を意味し、揺らぎ塩基対とは、グアニン-ウラシル、イノシン-ウラシル、イノシン-アデニン及びイノシン-シトシン間に水素結合が形成される塩基対を意味する。また、「相補的な塩基配列」とは、対象となる塩基配列と１００％の相補性を有していなくてもよく、例えば、対象となる塩基配列に対して、1～3個、1～2個又は1個の非相補的塩基が含まれていてもよい。

【００２１】

本明細書中、「ストリンジェントな条件」とは、低ストリンジェントな条件、中ストリンジェントな条件及び高ストリンジェントな条件のいずれでもよい。「低ストリンジェントな条件」は、例えば、5×SSC、5×デンハルト溶液、0.5％SDS、50％ホルムアミド、32℃の条件である。また、「中ストリンジェントな条件」は、例えば、5×SSC、5×デンハルト溶液、0.5％SDS、50％ホルムアミド、42℃又は5×SSC、1％ SDS、50 mM Tris－HCl（pH7.5）、50％ホルムアミド、42℃の条件である。「高ストリンジェントな条件」は、例えば、5×SSC、5×デンハルト溶液、0.5％SDS、50％ホルムアミド、50℃又は0.2×SSC、0.1％ SDS、65℃の条件である。これらの条件において、温度を上げるほど高い同一性を有するポリヌクレオチドが効率的に得られることが期待できる。ただし、ハイブリダイゼー

ションのストリンジェンシーに影響する要素としては温度、プローブ濃度、プローブの長さ、イオン強度、時間、塩濃度等の複数の要素が考えられ、当業者であればこれらの要素を適宜選択することで同様のストリンジェンシーを実現することが可能である。

【００２２】

なお、ハイブリダイゼーションに市販のキットを用いる場合は、例えばAlkphos Direct Labelling and Detection System（GE Healthcare）を用いることができる。この場合は、キットに添付のプロトコールにしたがい、標識したプローブとのインキュベーションを一晩行った後、メンブレンを55℃の条件下で0.1%（w/v）SDSを含む1次洗浄バッファーで洗浄後、ハイブリダイズしたポリヌクレオチドを検出することができる。あるいは、配列番号1の塩基配列と相補的な塩基配列の全部又は一部に基づいてプローブを作製する際に、市販の試薬（例えば、PCRラベリングミックス（ロシュ・ダイアグノス社）等）を用いて該プローブをジゴキシゲニン（DIG）ラベルした場合には、DIG核酸検出キット（ロシュ・ダイアグノス社）を用いてハイブリダイゼーションを検出することができる。

【００２３】

上記のハイブリダイズ可能なポリヌクレオチド以外のポリヌクレオチドとしては、相同性検索ソフトウェアであるBLASTにより、デフォルトのパラメーターを用いて計算したときに、配列番号1のポリヌクレオチドと90%以上、91%以上、92%以上、93%以上、94%以上、95%以上、96%以上、97%以上、98%以上、99%以上、99.1%以上、99.2%以上、99.3%以上、99.4%以上、99.5%以上、99.6%以上、99.7%以上、99.8%以上、又は99.9%以上の同一性を有するポリヌクレオチドをあげることができる。

【００２４】

なお、塩基配列の同一性は、カーリン及びアルチュールによるアルゴリズムBLAST（Basic Local Alignment Search Tool）（Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc Natl Acad Sci USA 90: 5873, 1993）を用いて決定できる。BLASTのアルゴリズムに基づいたBLASTNやBLASTXと呼ばれるプログラムが開発されている（Altschul SF, et al: J Mol Biol 215: 403, 1990）。BLASTNを用いて塩基配列を解析する場合は、パラメーターは、例えばscore = 100、wordlength = 12とする。BLASTとGapped BLASTプログラムを用いる場合は、各プログラムのデフォルトパラメーターを用いる。

【００２５】

エクソン53の5’末端から第31～53番目、第31～54番目、第31～55番目、第31～56番目、第31～57番目、第31～58番目、第32～53番目、第32～54番目、第32～55番目、第32～56番目、第32～57番目、第32～58番目、第33～53番目、第33～54番目、第33～55番目、第33～56番目、第33～57番目、第33～58番目、第34～53番目、第34～54番目、第34～55番目、第34～56番目、第34～57番目、第34～58番目、第35～53番目、第35～54番目、第35～55番目、第35～56番目、第35～57番目、第35～58番目、第36～53番目、第36～54番目、第36～55番目、第36～56番目、第36～57番目及び第36～58番目のヌクレオチドからなる配列に相補的な塩基配列の例を以下の表に示す。

【００２６】

【表１－Ａ】

表１

エクソン 53 中の標的配列	相補的な塩基配列	配列番号
第 31～53 番目	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 2
第 31～54 番目	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 3
第 31～55 番目	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 4
第 31～56 番目	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 5
第 31～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 6
第 31～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	配列番号 7
第 32～53 番目	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 8
第 32～54 番目	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 9
第 32～55 番目	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 10
第 32～56 番目	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 11
第 32～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 12
第 32～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	配列番号 13
第 33～53 番目	5'-CCGGTTCTGAAGGTGTTCTTG-3'	配列番号 14
第 33～54 番目	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	配列番号 15
第 33～55 番目	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	配列番号 16
第 33～56 番目	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	配列番号 17
第 33～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	配列番号 18
第 33～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	配列番号 19
第 34～53 番目	5'-CCGGTTCTGAAGGTGTTCTT-3'	配列番号 20
第 34～54 番目	5'-TCCGGTTCTGAAGGTGTTCTT-3'	配列番号 21
第 34～55 番目	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	配列番号 22
第 34～56 番目	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	配列番号 23
第 34～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	配列番号 24
第 34～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	配列番号 25
第 35～53 番目	5'-CCGGTTCTGAAGGTGTTCT-3'	配列番号 26
第 35～54 番目	5'-TCCGGTTCTGAAGGTGTTCT-3'	配列番号 27

【表１－Ｂ】

第 35～55 番目	5'-CTCCGGTTCTGAAGGTGTTCT-3'	配列番号 28
第 35～56 番目	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	配列番号 29
第 35～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	配列番号 30
第 35～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	配列番号 31
第 36～53 番目	5'-CCGGTTCTGAAGGTGTTC-3'	配列番号 32
第 36～54 番目	5'-TCCGGTTCTGAAGGTGTTC-3'	配列番号 33
第 36～55 番目	5'-CTCCGGTTCTGAAGGTGTTC-3'	配列番号 34
第 36～56 番目	5'-CCTCCGGTTCTGAAGGTGTTC-3'	配列番号 35
第 36～57 番目	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	配列番号 36
第 36～58 番目	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	配列番号 37

【００２７】

本発明のオリゴマーは、好ましくは、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第32～56番目、第33～56番目、第34～56番目、第35～56番目又は第36～56番目のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列（例えば、配列番号11、配列番号17、配列番号23、配列番号29又は配列番号35）からなる。

好ましくは、本発明のオリゴマーは、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第32～56番目又は第36～56番目のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列（例えば、配列番号11又は配列番号35）からなるものである。

【００２８】

「ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にする」とは、ヒトジストロフィン遺伝子の転写物（例えば、pre-mRNA）のエクソン53に相当する部位に本発明のオリゴマーが結合することにより、該転写物がスプライシングを受けた際に、例えばエクソン52が欠失したDMD患者の場合、エクソン51の3’末端に相当する塩基配列の3’側にエクソン54の5’末端に相当する塩基配列が連結し、コドンのフレームシフトが起こっていない成熟mRNAが形成されることを意味する。

従って、本発明のオリゴマーは、ヒトジストロフィン遺伝子のエクソン53のスキッピングを可能にする限り、ターゲット配列に対して100%相補的な塩基配列を有していなくてもよい。例えば、本発明のオリゴマーには、ターゲット配列に対して、1～3個、1～2個又は1個の非相補的塩基が含まれていてもよい。

ここで、前記「結合」は、本発明のオリゴマーとヒトジストロフィン遺伝子の転写物とを混合した場合に、生理的条件下で両者がハイブリダイズして二本鎖を形成することを意味する。上記「生理的条件下」とは、生体内と類似のpH、塩組成、温度に調節された条件を意味する。例えば、25～40℃、好ましくは37℃、pH 5～8、好ましくは、pH 7.4であって、塩化ナトリウム濃度が150 mMの条件が挙げられる。

【００２９】

ヒトジストロフィン遺伝子のエクソン53のスキッピングが生じたか否かは、ジストロフィン発現細胞（例えば、ヒト横紋筋肉腫細胞）に本発明のオリゴマーを導入し、前記ジストロフィン発現細胞のtotal RNAから、ヒトジストロフィン遺伝子のmRNAのエクソン53の周辺領域をRT-PCR増幅し、該PCR増幅産物に対してnested PCR又はシークエンス解析を行うことにより確認することができる。

スキッピング効率は、ヒトジストロフィン遺伝子のmRNAを被検細胞から回収し、該mRNAのうち、エクソン53がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定し、これら「A」及び「B」の測定値に基づき、以下の式に従って計算することができる。

スキッピング効率（%）= A /（ A ＋ B ）x 100

【００３０】
本発明のオリゴマーとしては、例えば、18～28 塩基の長さを有する、オリゴヌクレオチド、モルホリノオリゴマー、又はペプチド核酸（Peptide Nucleic Acid:PNA）オリゴマーを挙げることができる。21～25塩基の長さが好ましく、モルホリノオリゴマーが好ましい。
【００３１】
前記オリゴヌクレオチド（以下、「本発明のオリゴヌクレオチド」という）は、ヌクレオチドを構成単位とする本発明のオリゴマーであり、かかるヌクレオチドは、リボヌクレオチド、デオキシリボヌクレオチド又は修飾ヌクレオチドのいずれであってもよい。
修飾ヌクレオチドとは、リボヌクレオチド又はデオキシリボヌクレオチドを構成する核酸塩基、糖部分、及びリン酸結合部分の全部又は一部が修飾されているものをいう。
【００３２】
核酸塩基としては、例えば、アデニン、グアニン、ヒポキサンチン、シトシン、チミン、ウラシル又はそれらの修飾塩基を挙げることができる。かかる修飾塩基としては、例えば、シュードウラシル、3-メチルウラシル,ジヒドロウラシル、5-アルキルシトシン（例えば、5-メチルシトシン）、5-アルキルウラシル（例えば、5-エチルウラシル）、5-ハロウラシル（5-ブロモウラシル）、6-アザピリミジン、6-アルキルピリミジン（6-メチルウラシル）、2-チオウラシル、4-チオウラシル、4-アセチルシトシン、5-(カルボキシヒドロキシメチル）ウラシル、5'-カルボキシメチルアミノメチル-2-チオウラシル、5-カルボキシメチルアミノメチルウラシル、1-メチルアデニン、1-メチルヒポキサンチン、2,2-ジメチルグアニン、3-メチルシトシン、2-メチルアデニン、2-メチルグアニン、N6-メチルアデニン、7-メチルグアニン、5-メトキシアミノメチル-2-チオウラシル、5-メチルアミノメチルウラシル、5-メチルカルボニルメチルウラシル、5-メチルオキシウラシル、5-メチル-2-チオウラシル、2-メチルチオ-N6-イソペンテニルアデニン、ウラシル-5-オキシ酢酸、2-チオシトシン、プリン、2,6-ジアミノプリン、2-アミノプリン、イソグアニン、インドール、イミダゾール、キサンチン等が挙げられるが、これらに限定されるものではない。
【００３３】
糖部分の修飾としては、例えば、リボースの2'位の修飾及び糖のその他の部分の修飾を挙げることができる。リボースの2'位の修飾としては、例えば、リボースの2'位の-OH基をOR、R、R'OR、SH、SR、NH_2、NHR、NR_2、N_3、CN、F、Cl、Br、Iに置換する修飾を挙げることができる。ここで、Rはアルキル又はアリールを表す。R'はアルキレンを表す。
糖のその他の部分の修飾としては、例えば、リボース又はデオキシリボースの4'位のOをSに置換したもの、糖の 2'位と 4'位を架橋したもの、例えば、LNA（Locked Nucleic Acid）又はENA(2'-O,4'-C-Ethylene-bridged Nucleic Acids)などが挙げられるが、これらに限定されるものではない。
【００３４】
リン酸結合部分の修飾としては、例えば、ホスホジエステル結合をホスホロチオエート結合、ホスホロジチオエート結合、アルキルホスホネート結合、ホスホロアミデート結合、ボラノフォスフェート結合（Enya et al: Bioorganic & Medicinal Chemistry ,2008, 18, 9154-9160 ）に置換する修飾を挙げることができる（例えば、特許再公表公報第2006/129594号及び第2006/038608号を参照）。
【００３５】

アルキルとしては、直鎖状または分枝鎖状の炭素数1～6のアルキルが好ましい。具体的には、例えば、メチル、エチル、n-プロピル、イソプロピル、n-ブチル、イソブチル、sec-ブチル、tert-ブチル、n-ペンチル、イソペンチル、ネオペンチル、tert-ペンチル、n-ヘキシル、イソヘキシルが挙げられる。当該アルキルは置換されていてもよく、かかる置換基としては、例えば、ハロゲン、アルコキシ、シアノ、ニトロを挙げることができ、これらが1～3個置換されていてもよい。

【００３６】

シクロアルキルとしては、炭素数5～12のシクロアルキルが好ましい。具体的には、例えば、シクロペンチル、シクロヘキシル、シクロヘプチル、シクロオクチル、シクロデシル、シクロドデシルが挙げられる。

【００３７】

ハロゲンとしては、フッ素、塩素、臭素、ヨウ素を挙げることができる。

【００３８】

アルコキシとしては、直鎖状または分枝鎖状の炭素数1～6のアルコキシ、例えば、メトキシ、エトキシ、n-プロポキシ、イソプロポキシ、n-ブトキシ、イソブトキシ、sec-ブトキシ、tert-ブトキシ、n-ペンチルオキシ、イソペンチルオキシ、n-ヘキシルオキシ、イソヘキシルオキシ等を挙げることができる。とりわけ、炭素数1～3のアルコキシが好ましい。

【００３９】

アリールとしては、炭素数6～10のアリールが好ましい。具体的には、例えば、フェニル、α-ナフチル、β-ナフチルを挙げることができる。とりわけフェニルが好ましい。当該アリールは置換されていてもよく、かかる置換基としては、例えば、アルキル、ハロゲン、アルコキシ、シアノ、ニトロを挙げることができ、これらが1～3個置換されていてもよい。

【００４０】

アルキレンとしては、直鎖状または分枝鎖状の炭素数1～6のアルキレンが好ましい。具体的には、例えば、メチレン、エチレン、トリメチレン、テトラメチレン、ペンタメチレン、ヘキサメチレン、2-（エチル）トリメチレン、1-（メチル）テトラメチレンを挙げることができる。

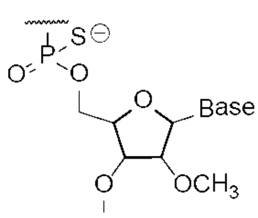
【００４１】

アシルとしては、直鎖状若しくは分枝鎖状のアルカノイル、又はアロイルを挙げることができる。アルカノイルとしては、例えば、ホルミル、アセチル、２－メチルアセチル、２，２－ジメチルアセチル、プロピオニル、ブチリル、イソブチリル、ペンタノイル、２，２－ジメチルプロピオニル、ヘキサノイル等が挙げられる。アロイルとしては、例えば、ベンゾイル、トルオイル、ナフトイルを挙げることができる。かかるアロイルは置換可能な位置において置換されていてもよく、アルキルで置換されていてもよい。

【００４２】

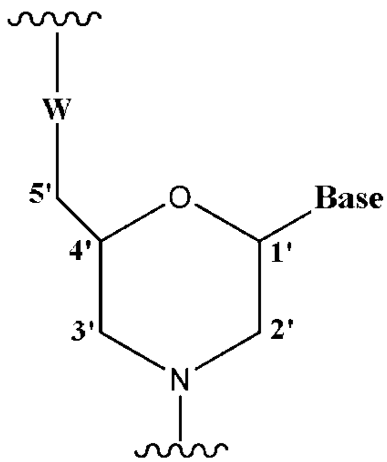
本発明のオリゴヌクレオチドは、好ましくは、リボースの2’位の-OH基がメトキシで置換され、リン酸結合部分がホスホロチオエート結合である、下記一般式で表される基を構成単位とする本発明のオリゴマーである。

【化２】

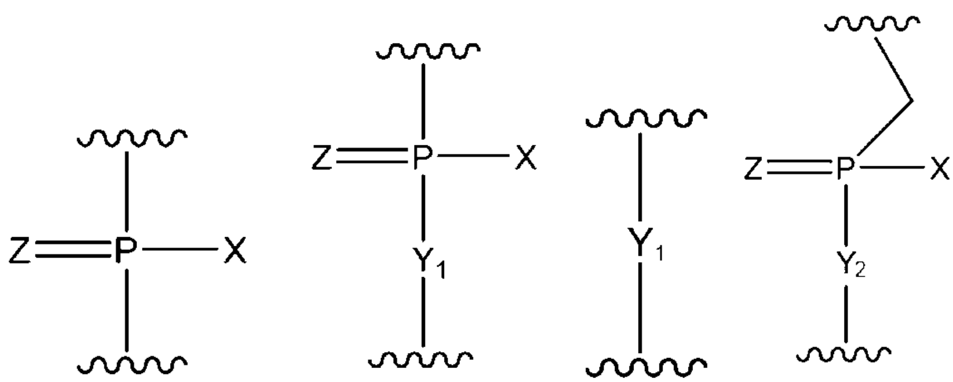


（式中、Baseは、核酸塩基を表す。）

【００４３】
本発明のオリゴヌクレオチドは、各種自動合成装置（例えば、AKTA oligopilot plus 10 / 100（GE Healthcare））を用いて容易に合成することが可能であり、あるいは、第三者機関（例えば、Promega社又はTakara社）等に委託して作製することもできる。
【００４４】
本発明のモルホリノオリゴマーは、下記一般式で表される基を構成単位とする本発明のオリゴマーである。
【化３】

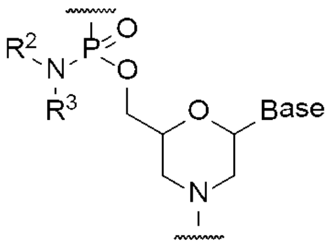


（式中、Baseは、前記と同義であり；
Wは、以下のいずれかの式で表わされる基を表す。
【化４】

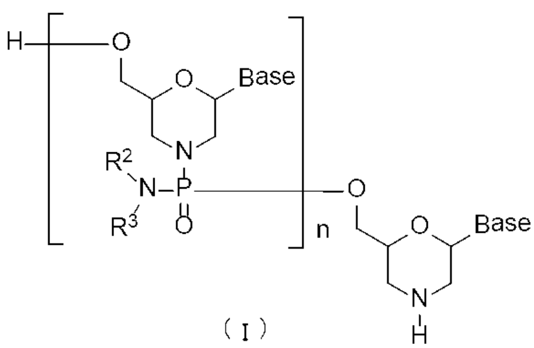


(式中、Xは、$-CH_2R^1$、$-O-CH_2R^1$、$-S-CH_2R^1$、$-NR^2R^3$又はFを表し；
R^1は、H、アルキルを表し；
R^2及びR^3は、同一又は異なって、H、アルキル、シクロアルキル、又は、アリールを表し；
Y_1は、O、S、CH_2又はNR^1を表し；
Y_2は、O、S又はNR^1を表し；
Zは、O又はSを表す。))

【００４５】
モルホリノオリゴマーは、好ましくは、以下の式で表わされる基を構成単位とするオリゴマー（ホスホロジアミデートモルホリノオリゴマー（以下、「PMO」という））である。
【化５】

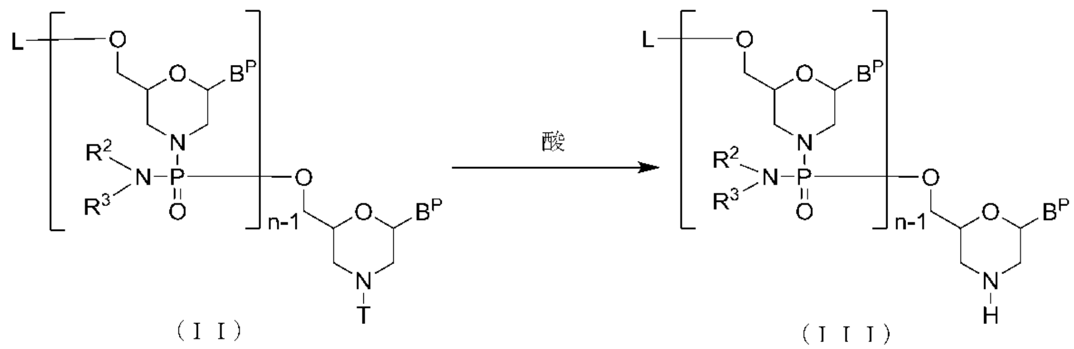


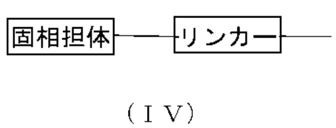
（式中、Base、R^2、R^3は、前記と同義である。）
【００４６】
モルホリノオリゴマーは、例えば、国際公開公報第1991/009033号、又は国際公開公報第2009/064471号に従って製造することができる。特に、PMOは、国際公開公報第2009/064471号に記載の方法に従って製造するか、又は以下に示す方法に従って製造することができる。
【００４７】
[PMOの製法]
PMOの１つの態様として、例えば、次の一般式(I)で表される化合物（以下、PMO(I)という）を挙げることができる。
【化６】



（I）

[式中、各Base、R^2、R^3は、前記と同義であり；
nは、1〜99の範囲内にある任意の整数であり、好ましくは、18〜28の範囲内にある任意の整数である。]
【００４８】
PMO（I）は、公知の方法に従い製造することができるが、例えば、下記工程の操作を実施することにより製造することができる。
下記工程に使用されている化合物及び試薬は、PMOの製造に一般的に使用されているものであれば特に限定されない。
【００４９】
また、下記のすべての工程は、液相法又は固相法（マニュアル又は市販の固相自動合成機を用いる）で実施することができる。固相法でPMOを製造する場合、操作手順の簡便化及び合成の正確性の点から自動合成機を用いる方法が望ましい。
【００５０】

（１）工程Ａ：
次の一般式（II）で表される化合物（以下、化合物（II）という）に酸を作用させることによって、次の一般式（III）で表される化合物（以下、化合物（III）という。）を製造する工程。
【化７】


［式中、n、R^2、R^3は、前記と同義であり；
各B^Pは，独立して、保護されていてもよい核酸塩基を表し；
Tは、トリチル基、モノメトキシトリチル基、又はジメトキシトリチル基を表し；
Lは、水素、アシル、又は次の一般式（IV）で表される基（以下、基（IV）という。）を表す。］
【化８】


【００５１】
B^Pに係る「核酸塩基」としては、Baseと同じ「核酸塩基」を挙げることができる。但し、B^Pに係る核酸塩基のアミノ基又は水酸基は保護されていてもよい。
かかるアミノ基の保護基としては、核酸の保護基として使用されるものであれば特に制限されず、具体的には、例えば、ベンゾイル、4-メトキシベンゾイル、アセチル、プロピオニル、ブチリル、イソブチリル、フェニルアセチル、フェノキシアセチル、4-tert-ブチルフェノキシアセチル、4-イソプロピルフェノキシアセチル、(ジメチルアミノ)メチレンを挙げることができる。水酸基の保護基としては、例えば、2-シアノエチル、４－ニトロフェネチル、フェニルスルホニルエチル、メチルスルホニルエチル、トリメチルシリルエチル、置換可能な任意の位置で1～5個の電子吸引性基で置換されていてもよいフェニル、ジフェニルカルバモイル、ジメチルカルバモイル、ジエチルカルバモイル、メチルフェニルカルバモイル、1-ピロリジニルカルバモイル、モルホリノカルバモイル、4-（tert-ブチルカルボキシ）ベンジル、4-[(ジメチルアミノ)カルボキシ]ベンジル、4-(フェニルカルボキシ)ベンジルを挙げることができる（例えば、国際公開公報第2009/064471号公報参照）。
【００５２】
「固相担体」としては、核酸の固相反応に使用しうる担体であれば特に制限されないが、例えば、（ｉ）モルホリノ核酸誘導体の合成に使用しうる試薬（例えば、ジクロロメタン、アセトニトリル、テトラゾール、N-メチルイミダゾール、ピリジン、無水酢酸、ルチジン、トリフルオロ酢酸）にほとんど溶解せず、（ｉｉ）モルホリノ核酸誘導体の合成に使用しうる試薬に対して化学的に安定であり、（ｉｉｉ）化学修飾ができ、（ｉｖ）望ましいモルホリノ核酸誘導体の装填ができ、（ｖ）処理中にかかる高圧に耐える十分な強度をもち

、（vi）一定の粒径範囲と分布であるものが望ましい。具体的には、膨潤性ポリスチレン（例えば、アミノメチルポリスチレン樹脂 1%ジベンジルベンゼン架橋（200～400メッシュ）（2.4～3.0mmol/g）（東京化成社製）、Aminomethylated Polystyrene Resin・HCl [ジベンジルベンゼン1%, 100～200メッシュ]（ペプチド研究所社製））、非膨潤性ポリスチレン（例えば、Primer Support(GE Healthcare社製））、PEG鎖結合型ポリスチレン（例えば、NH_2-PEG resin（渡辺化学社製）、TentaGel resin）、定孔ガラス（controlled pore glass;CPG）（例えば、CPG社製）、オキサリル化ー定孔ガラス（例えば、Alulら, Nucleic Acids Research,Vol.19,1527(1991)を参照）、TentaGel支持体ーアミノポリエチレングリコール誘導体化支持体（例えば、Wrightら, Tetrahedron Letters,Vol.34,3373(1993)を参照）、Poros-ポリスチレン/ジビニルベンゼンのコポリマーを挙げることができる。

【００５３】

「リンカー」としては、通常核酸やモルホリノ核酸誘導体を連結するために使用される公知のものを用いることができるが、例えば、3-アミノプロピル、スクシニル、2,2'-ジエタノールスルホニル、ロングチェーンアルキルアミノ（LCAA）を挙げることができる。

【００５４】

本工程は、化合物（II）に酸を作用させることにより実施することができる。

本工程に使用しうる「酸」としては、例えば、トリフルオロ酢酸、ジクロロ酢酸又はトリクロロ酢酸を挙げることができる。酸の使用量としては、例えば、化合物（II）1モルに対して0.1モル当量～1000モル当量の範囲内が適当であり、好ましくは1モル当量～100モル当量の範囲内である。

また、前記酸と一緒に、有機アミンを使用することができる。有機アミンとしては、特に限定されるものではないが、例えば、トリエチルアミンを挙げることができる。有機アミンの使用量は、例えば、酸1モルに対して、0.01モル当量～10モル当量の範囲内が適当であり、好ましくは、0.1モル当量～2モル当量の範囲内である。

【００５５】

本工程において酸と有機アミンとの塩又は混合物を使用する場合には、例えば、トリフルオロ酢酸とトリエチルアミンの塩又は混合物を挙げることができ、より具体的には、トリフルオロ酢酸2当量に対してトリエチルアミン1当量を混合したものを挙げることができる。

本工程に使用しうる酸は、0.1%～30%の範囲内の濃度になるように適当な溶媒で希釈して使用することもできる。溶媒としては、反応に関与しなければ特に限定されないが、例えば、ジクロロメタン、アセトニトリル、アルコール類（エタノール、イソプロパノール、トリフルオロエタノールなど）、水又はこれらの混合物を挙げることができる。

【００５６】

上記反応における反応温度は、例えば、10℃～50℃の範囲内が好ましく、より好ましくは、20℃～40℃の範囲内であり、さらに好ましくは、25℃～35℃の範囲内である。

反応時間は、使用する酸の種類、反応温度によって異なるが、通常0.1分～24時間の範囲内が適当である。好ましくは、1分～5時間の範囲内である。

【００５７】

また、本工程が終了した後、必要に応じて、系中に存在する酸を中和するために塩基を添加することができる。「塩基」としては、特に限定されないが、例えば、ジイソプロピルアミンが挙げられる。塩基は、0.1%（v/v）～30%（v/v）の範囲内の濃度になるように適当な溶媒で希釈して使用することもできる。

本工程に用いる溶媒としては、反応に関与しなければ特に限定されないが、ジクロロメタン、アセトニトリル、アルコール類（エタノール、イソプロパノール、トリフルオロエタノールなど）、水又はこれらの混合物を挙げることができる。反応温度は、例えば、10℃～50℃の範囲内が好ましく、より好ましくは、20℃～40℃の範囲内であり、さらに好ましくは、25℃～35℃の範囲内である。

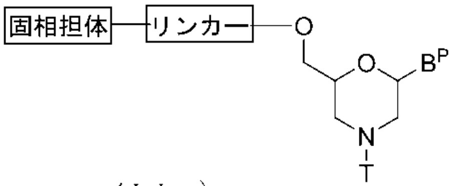
反応時間は、使用する塩基の種類、反応温度によって異なるが、通常0.1分～24時間の

範囲内が適当であり、好ましくは、1分～5時間の範囲内である。

【００５８】

なお、化合物（II）において、n=1であって、Lが基（IV）である、次の一般式（IIa）で表される化合物（以下、化合物（IIa）という）は、以下の方法に従って製造することができる。

【化９】



（ＩＩａ）

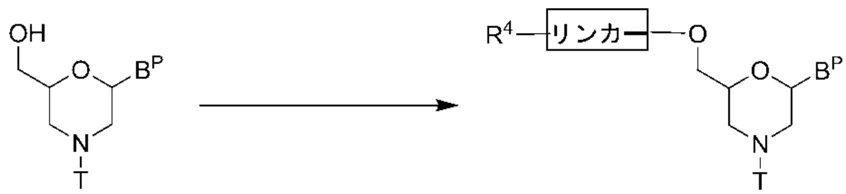
［式中、B^P、T、リンカー、固相担体は、前記と同義である。］

【００５９】

工程１：

次の一般式（V）で表される化合物にアシル化剤を作用させることによって、次の一般式（VI）で表される化合物（以下、化合物（VI）という。）を製造する工程。

【化１０】



（Ｖ） （ＶＩ）

［式中、B^P、T、リンカーは、前記と同義であり；

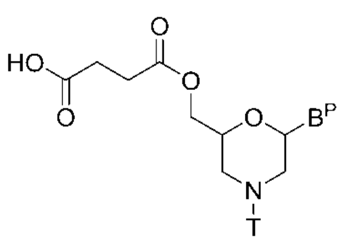
R^{4}は、水酸基、ハロゲン、又は、アミノを表す。］

【００６０】

本工程は、化合物（V）を出発原料として、公知のリンカーの導入反応により実施することができる。

特に、次の一般式（VIa）で表される化合物は、化合物（V）と無水コハク酸とを用いてエステル化反応として知られた方法を実施することにより製造することができる。

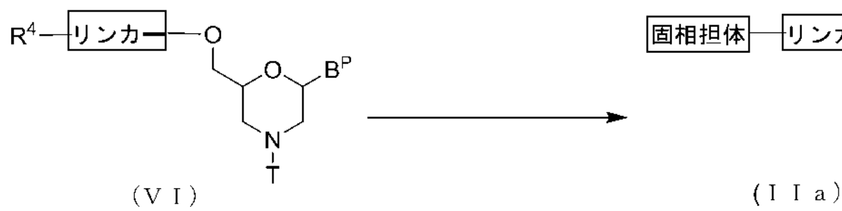
【化１１】



（ＶＩａ）

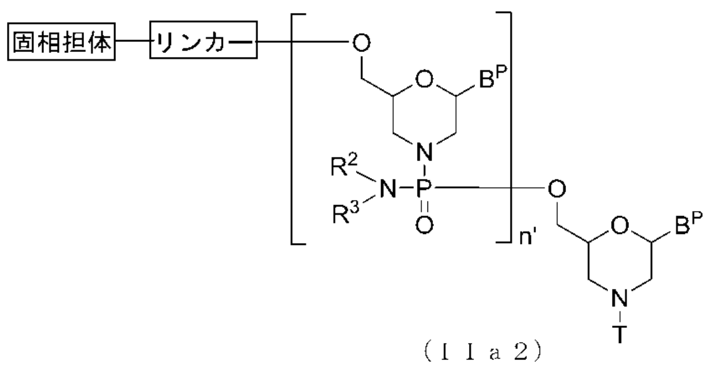
［式中、B^P、Tは、前記と同義である。］

【００６１】
工程２：
　化合物（VI）に縮合剤等を作用させることによって、固相担体と反応させ、化合物（IIa）を製造する工程。
【化１２】



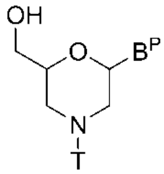
［式中、B^P、R^4、T、リンカー、固相担体は、前記と同義である。］

【００６２】
　本工程は、化合物(VI)と固相担体とを用いて縮合反応として知られた方法により製造することができる。
【００６３】
　化合物(II)において、n=2～99であって、Lが基(IV)である、次の一般式(IIa2)で表される化合物は、化合物(IIa)を出発原料とし、本明細書に記載のPMOの製法にかかる工程A及び工程Bを所望の回数繰り返し実施することにより製造することができる。
【化１３】



［式中、B^P、R^2、R^3、T、リンカー、固相担体は、前記と同義であり；
　n’は、1～98を表す。］
【００６４】
　また、化合物(II)において、n=1であって、Lが水素である、次の一般式(IIb)で表される化合物は、例えば、国際公開公報第1991/009033号に記載の方法により製造することができる。

【化１４】



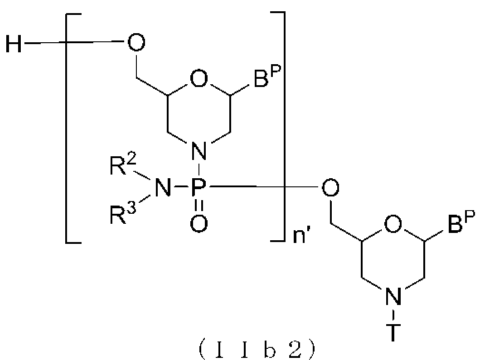
（ＩＩｂ）

［式中、B^P、Tは、前記と同義である。］

【００６５】

化合物（ＩＩ）において、n=2～99であって、Lが水素である、次の一般式（ＩＩb2）で表される化合物は、化合物（ＩＩb）を出発原料とし、本明細書に記載のPMOの製法にかかる工程A及び工程Bを所望の回数繰り返し実施することにより製造することができる。

【化１５】



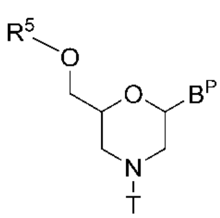
（ＩＩｂ２）

［式中、B^P、n’、R^2、R^3、Tは、前記と同義である。］

【００６６】

また、化合物（ＩＩ）において、n=1であって、Lがアシルである、次の一般式（ＩＩc）で表される化合物は、化合物（ＩＩb）に対してアシル化反応として知られた方法を実施することにより製造することができる。

【化１６】



（ＩＩｃ）

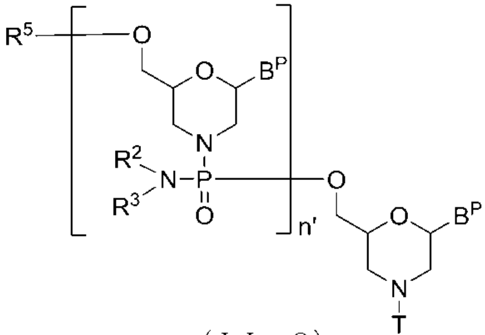
［式中、B^P、Tは、前記と同義であり；

R^5は、アシルを表す。］

【００６７】

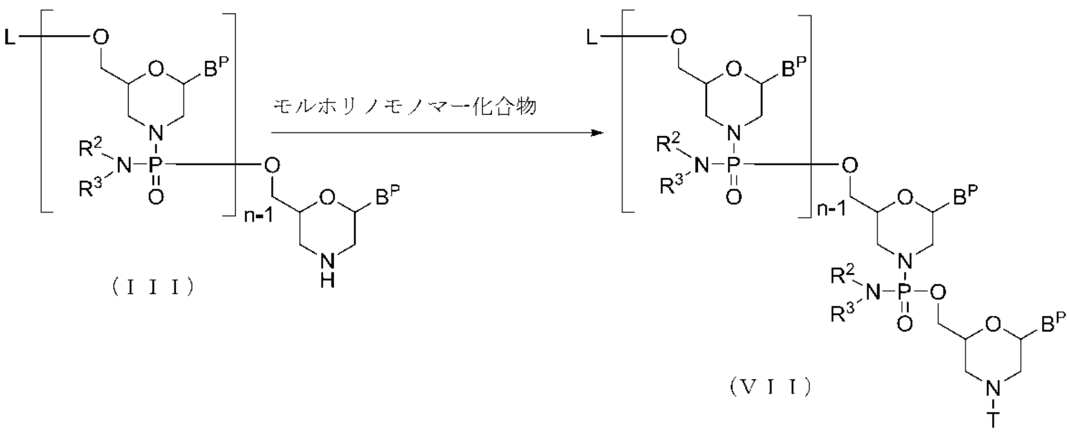
化合物（ＩＩ）において、n=2～99であって、Lがアシルである、次の一般式（ＩＩc2）で表される化合物は、化合物（ＩＩc）を出発原料とし、本明細書に記載のPMOの製法にかかるエ

程A及び工程Bを所望の回数繰り返し実施することにより製造することができる。
【化１７】



（ＩＩｃ２）

［式中、B^P、n’、R^2、R^3、R^5、Tは、前記と同義である。］
【００６８】
（２）工程B：
化合物（III）に塩基存在下にモルホリノモノマー化合物を作用させることによって、次の一般式（VII）で表される化合物（以下、化合物（VII）という。）を製造する工程。
【化１８】



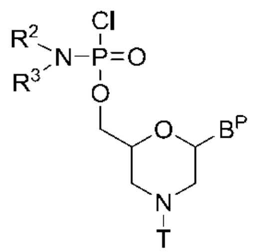
（ＩＩＩ）

（ＶＩＩ）

［式中、各B^P、L、n、R^2、R^3、Tは、前記と同義である。］

【００６９】
本工程は、化合物（III）に塩基存在下にモルホリノモノマー化合物を作用させることにより実施することができる。
【００７０】
モルホリノモノマー化合物としては、例えば、次の一般式（VIII）で表される化合物を挙げることができる。

【化１９】



（ＶＩＩＩ）

［式中、B^P、R^2、R^3、Tは前記と同義である。］

【００７１】

本工程に使用しうる「塩基」としては、例えば、ジイソプロピルアミン、トリエチルアミン、又は、N-エチルモルホリンを挙げることができる。塩基の使用量としては、例えば、化合物（III）1モルに対して、1モル当量～1000モル当量の範囲内が適当であり、好ましくは10モル当量～100モル当量の範囲内である。

本工程に使用しうるモルホリノモノマー化合物および塩基は、0.1%～30%の濃度になるように適当な溶媒で希釈して使用することもできる。溶媒としては、反応に関与しなければ特に限定されないが、例えば、N,N-ジメチルイミダゾリドン、N-メチルピペリドン、DMF、ジクロロメタン、アセトニトリル、テロラヒドロフラン、又はこれらの混合物を挙げることができる。

【００７２】

反応温度は、例えば、0℃～100℃の範囲内が好ましく、より好ましくは、10℃～50℃の範囲内である。

反応時間は、使用する塩基の種類、反応温度によって異なるが、通常1分～48時間の範囲内が適当であり、好ましくは、30分～24時間の範囲内である。

【００７３】

さらに本工程の終了後、必要に応じて、アシル化剤を添加することができる。「アシル化剤」としては、例えば、無水酢酸、酢酸クロライド、フェノキシ酢酸無水物を挙げることができる。アシル化剤は、例えば、0.1%～30%の範囲内の濃度になるように適当な溶媒で希釈して使用することもできる。溶媒としては、反応に関与しなければ特に限定されないが、例えば、ジクロロメタン、アセトニトリル、アルコール類（エタノール、イソプロパノール、トリフルオロエタノールなど）、水又はこれらの混合物を挙げることができる。

また、必要であれば、アシル化剤と一緒に、例えば、ピリジン、ルチジン、コリジン、トリエチルアミン、ジイソプロピルエチルアミン、N-エチルモルホリン等の塩基を使用することができる。アシル化剤の使用量としては、0.1モル当量～10000モル当量の範囲内が好ましく、1モル当量～1000モル当量の範囲内がより好ましい。塩基の使用量としては、例えば、アシル化剤１モルに対して、0.1モル当量～100モル当量の範囲内が適当であり、好ましくは1モル当量～10モル当量の範囲内である。

【００７４】

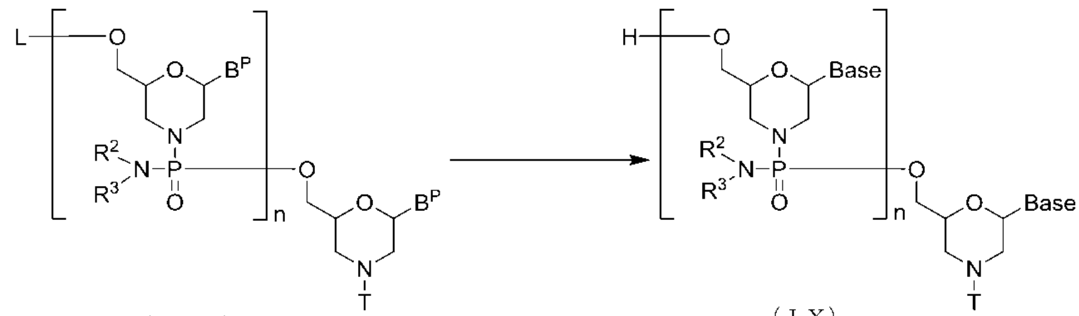
本反応の反応温度は、10℃～50℃の範囲内が好ましく、より好ましくは、10℃～50℃の範囲内が好ましく、より好ましくは、20℃～40℃の範囲内であり、さらに好ましくは、25℃～35℃の範囲内である。反応時間は、例えば、使用するアシル化剤の種類、反応温度によって異なるが、通常0.1分～24時間の範囲内が適当であり、好ましくは、1分から5時間の範囲内である。

【００７５】

（３）工程C：

工程Bにおいて製造される化合物（VII）において、脱保護剤を用いて保護基を脱離し、一般式（IX）で表される化合物を製造する工程。

【化２０】



（ＶＩＩ）　（ＩＸ）

［式中、Base、B^P、L、n、R^2、R^3、Tは、前記と同義である。］

【００７６】

本工程は、化合物（VII）に脱保護剤を作用させることにより実施することができる。

【００７７】

「脱保護剤」としては、例えば、濃アンモニア水、メチルアミンを挙げることができる。本工程に使用しうる「脱保護剤」は、例えば、水、メタノール、エタノール、イソプロピルアルコール、アセトニトリル、テトラヒドロフラン、DMF、N,N-ジメチルイミダゾリドン、N-メチルピペリドン又はこれらの混合溶媒で希釈して使用することもできる。なかでも、エタノールが好ましい。脱保護剤の使用量としては、例えば、化合物（VII）1モルに対して、例えば、1モル当量～100000モル当量の範囲内が適当であり、好ましくは10モル当量～1000モル当量の範囲内である。

【００７８】

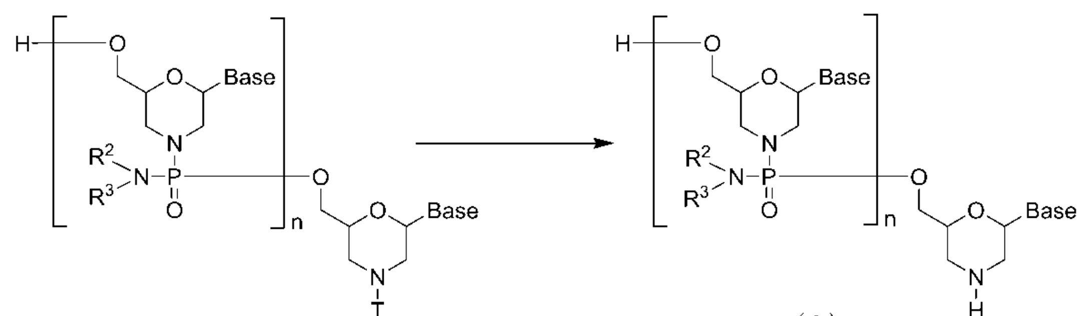
反応温度は、例えば、15℃～75℃の範囲内が適当であり、好ましくは40℃～70℃の範囲内であり、より好ましくは50℃～60℃の範囲内である。脱保護反応時間は、化合物（VII）の種類、反応温度等によって異なるが、10分～30時間の範囲内が適当であり、好ましくは30分～24時間の範囲内であり、より好ましくは5時間～20時間の範囲内である。

【００７９】

（４）工程D：

工程Cにおいて製造される化合物（IX）に酸を作用させることによって、PMO（I）を製造する工程。

【化２１】



（ＩＸ）　（Ｉ）

［式中、Base、n、R^2、R^3、Tは、前記と同義である。］

【００８０】

本工程は、化合物（ＩＸ）に酸を加えることによって実施することができる。

【００８１】

本工程において使用しうる「酸」としては、例えば、トリクロロ酢酸、ジクロロ酢酸、酢酸、リン酸及び塩酸等を挙げることができる。酸の使用量としては、例えば、溶液のpHが0.1～4.0の範囲内になるように使用するのが適当であり、より好ましくは1.0～3.0の範囲内になるように使用する。溶媒としては、反応に関与しなければ特に限定されないが、例えば、アセトニトリル、水、又はこれらの混合溶媒を挙げることができる。

【００８２】

反応温度は、10℃～50℃の範囲内が好ましく、より好ましくは、20℃～40℃の範囲内であり、さらに好ましくは、25℃～35℃の範囲内である。脱保護反応時間は、化合物（ＩＸ）の種類、反応温度等によって異なるが、0.1分～5時間の範囲内が適当であり、好ましくは1分～1時間の範囲内であり、より好ましくは1分～30分の範囲内である。

【００８３】

PMO(I)は、本工程で得られた反応混合物から通常の分離精製手段、例えば、抽出、濃縮、中和、濾過、遠心分離、再結晶、C_8からC_{18}の逆相カラムクロマトグラフィー、陽イオン交換カラムクロマトグラフィー、陰イオン交換カラムクロマトグラフィー、ゲルろ過カラムクロマトグラフィー、高速液体クロマトグラフィー、透析、限界ろ過などの手段を単独若しくは組み合わせて用いることにより得ることができ、所望のPMO(I)を単離精製することができる（例えば、国際公開公報WO1991/09033を参照）。

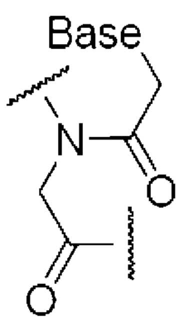
逆相クロマトグラフィーを用いてPMO(I)を精製する場合には、溶出溶媒として、例えば20mMのトリエチルアミン／酢酸緩衝液とアセトニトリルの混合溶液を使用することができる。

また、イオン交換クロマトグラフィーを用いてPMO(I)を精製する場合には、例えば、1Mの食塩水と10mMの水酸化ナトリウム水溶液の混合溶液を使用することができる。

【００８４】

ペプチド核酸は、下記一般式で表される基を構成単位とする本発明のオリゴマーである。

【化２２】



（式中、Baseは、前記と同義である。）

【００８５】

ペプチド核酸は、例えば、以下の文献に従って製造することができる。

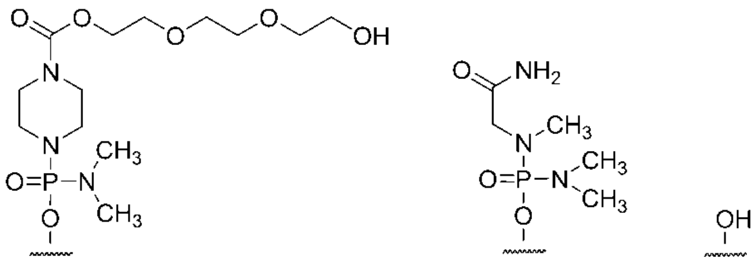
１）P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
２）M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
３）K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
４）L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

５）T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

【００８６】

また、本発明のオリゴマーは、5’末端が、下記化学式（1）～（3）のいずれかの基であってもよい。好ましくは(3)-OHである。

【化２３】



（１） （２） （３）

以下、上記(1)、(2)及び(3)で示される基を、それぞれ「基(1)」、「基(2)」及び「基(3)」と呼ぶ。

【００８７】

2. 医薬組成物

本発明のオリゴマーは、従来技術に係るアンチセンスオリゴマーと比較して、高効率にエクソン53のスキッピングを可能にする。従って、本発明のオリゴマーを含む医薬組成物をDMD患者に投与することにより、高効率に筋ジストロフィーの症状を緩和することができると予測される。例えば、本発明のオリゴマーを含む医薬組成物を用いる場合、従来技術に係るオリゴマーと比べて少量の投与量でも同程度の治療効果を得られるため、副作用を軽減することができ、かつ経済的である。

そこで、別の実施態様として、本発明のオリゴマー、その医薬的に許容可能な塩又は水和物を有効成分とする、筋ジストロフィー治療用医薬組成物（以下、「本発明の組成物」という）を提供する。

【００８８】

本発明の組成物に含まれる本発明のオリゴマーの医薬的に許容可能な塩の例としては、ナトリウム塩、カリウム塩、リチウム塩のようなアルカリ金属塩、カルシウム塩、マグネシウム塩のようなアルカリ土類金属塩；アルミニウム塩、鉄塩、亜鉛塩、銅塩、ニッケル塩、コバルト塩などの金属塩;アンモニウム塩；t-オクチルアミン塩、ジベンジルアミン塩、モルホリン塩、グルコサミン塩、フェニルグリシンアルキルエステル塩、エチレンジアミン塩、N-メチルグルカミン塩、グアニジン塩、ジエチルアミン塩、トリエチルアミン塩、ジシクロヘキシルアミン塩、N, N’-ジベンジルエチレンジアミン塩、クロロプロカイン塩、プロカイン塩、ジエタノールアミン塩、N-ベンジル-フェネチルアミン塩、ピペラジン塩、テトラメチルアンモニウム塩、トリス(ヒドロキシメチル)アミノメタン塩のような有機アミン塩;弗化水素酸塩、塩酸塩、臭化水素酸塩、沃化水素酸塩のようなハロゲン化水素酸塩；硝酸塩、過塩素酸塩、硫酸塩、リン酸塩などの無機酸塩;メタンスルホン酸塩、トリフルオロメタンスルホン酸塩、エタンスルホン酸塩のような低級アルカンスルホン酸塩；ベンゼンスルホン酸塩、p-トルエンスルホン酸塩のようなアリールスルホン酸塩；酢酸塩、りんご酸塩、フマール酸塩、コハク酸塩、クエン酸塩、酒石酸塩、シュウ酸塩、マレイン酸塩などの有機酸塩;グリシン塩、リジン塩、アルギニン塩、オルニチン塩、グルタミン酸塩、アスパラギン酸塩のようなアミノ酸塩などが挙げられる。これらの塩は、公知の方法で製造することができる。あるいは、本発明の組成物に含まれる本発明のオリゴマーは、その水和物の形態にあってもよい。

【００８９】
本発明の組成物の投与形態は、医薬的に許容可能な投与形態であれば特に制限されず、治療方法に応じて選択することができるが、筋組織への送達容易性の観点から、静脈内投与、動脈内投与、筋肉内投与、皮下投与、経口投与、組織内投与、経皮投与等が好ましい。また、本発明の組成物が取り得る剤型としては、特に制限されないが、例えば、各種の注射剤、経口剤、点滴剤、吸入剤、軟膏剤、ローション剤等を挙げることができる。
【００９０】
本発明のオリゴマーを筋ジストロフィー患者に投与する場合、本発明の組成物は、該オリゴマーの筋組織への送達を促進する担体を含むことが好ましい。このような担体は、医薬的に許容可能なものであれば特に制限されず、その例として、カチオン性リポソーム、カチオン性ポリマー等のカチオン性担体、またはウイルスエンベロープを利用した担体を挙げることができる。カチオン性リポソームとしては、例えば、2-O-（2-ジエチルアミノエチル）カルバモイル-1,3-O-ジオレオイルグリセロールとリン脂質とを必須構成成分として形成されるリポソーム（以下、「リポソームA」という）、オリゴフェクトアミン（登録商標）（Invitrogen社製）、リポフェクチン（登録商標）（Invitrogen社製）、リポフェクトアミン（登録商標）（Invitrogen社製）、Lipofectamine 2000（登録商標）（Invitrogen社製）、DMRIE-C（登録商標）（Invitrogen社製）、GeneSilencer（登録商標）（Gene Therapy Systems社製）、TransMessenger（登録商標）（QIAGEN社製）、TransIT TKO（登録商標）（Mirus社製）、Nucleofector II（Lonza）を挙げることができる。それらの中で、リポソームAが好ましい。カチオン性ポリマーとしては、例えば、JetSI（登録商標）（Qbiogene社製）、Jet-PEI（登録商標）（ポリエチレンイミン、Qbiogene社製）を挙げることができる。ウイルスエンベロープを利用した担体としては、例えば、Genome One（登録商標）（HVJ-Eリポソーム、石原産業社製）を挙げることができる。あるいは、特許2924179号に記載の医薬デバイス、特許再公表公報第2006/129594号及び特許再公表公報第2008/096690号に記載のカチオン性担体を用いることもできる。
【００９１】
本発明の組成物に含まれる本発明のオリゴマーの濃度は、担体の種類等によって異なるが、0.1 nM～100 μMの範囲内が適当であり、1 nM～10 μMの範囲内が好ましく、10 nM～1 μMの範囲内がより好ましい。また、本発明の組成物に含まれる本発明のオリゴマーと担体との重量比（担体/本発明のオリゴマー）は、該オリゴマーの性質及び該担体の種類等によって異なるが、0.1～100の範囲内が適当であり、1～50の範囲内が好ましく、10～20の範囲内がより好ましい。
【００９２】
本発明の組成物には、本発明のオリゴマーと上述した担体以外に、任意に医薬的に許容可能な添加剤を配合することができる。かかる添加剤として、例えば、乳化補助剤（例えば、炭素数6～22の脂肪酸やその医薬的に許容可能な塩、アルブミン、デキストラン）、安定化剤（例えば、コレステロール、ホスファチジン酸）、等張化剤（例えば、塩化ナトリウム、グルコース、マルトース、ラクトース、スクロース、トレハロース）、pH調整剤（例えば、塩酸、硫酸、リン酸、酢酸、水酸化ナトリウム、水酸化カリウム、トリエタノールアミン）を挙げることができる。これらを一種又は二種以上使用することができる。本発明の組成物中の当該添加剤の含有量は、90重量％以下が適当であり、70重量％以下が好ましく、50重量％以下がより好ましい。
【００９３】
本発明の組成物は、担体の分散液に本発明のオリゴマーを加え、適当に攪拌することにより調製することができる。また、添加剤は、本発明のオリゴマーの添加前でも添加後でも適当な工程で添加することができる。本発明のオリゴマーを添加させる際に用い得る水性溶媒としては、医薬的に許容可能なものであれば特に制限されず、例えば、注射用水、注射用蒸留水、生理食塩水等の電解質液、ブドウ糖液、マルトース液等の糖液を挙げることができる。また、かかる場合のpH及び温度等の条件は、当業者が適宜選択することができる。

【００９４】

本発明の組成物は、例えば、液剤やその凍結乾燥製剤とすることができる。当該凍結乾燥製剤は、常法により、液剤の形態を有している本発明の組成物を凍結乾燥処理することにより調製することができる。例えば、液剤の形態を有している本発明の組成物を適当な滅菌を行った後、所定量をバイアル瓶に分注し、約－40～－20℃の条件で予備凍結を2時間程度行い、約0～10℃で減圧下に一次乾燥を行い、次いで、約15～25℃で減圧下に二次乾燥して凍結乾燥することができる。そして、一般的にはバイアル内部を窒素ガスで置換し、打栓して本発明の組成物の凍結乾燥製剤を得ることができる。

【００９５】

本発明の組成物の凍結乾燥製剤は、一般には任意の適当な溶液（再溶解液）の添加によって再溶解し使用することができる。このような再溶解液としては、注射用水、生理食塩水、その他一般輸液を挙げることができる。この再溶解液の液量は、用途等によって異なり特に制限されないが、凍結乾燥前の液量の0.5～2倍量、又は500 mL以下が適当である。

【００９６】

本発明の組成物を投与する際の用量としては、含有される本発明のオリゴマーの種類、剤形、年齢や体重等の患者の状態、投与経路、疾患の性質と程度を考慮した上で調製することが望ましいが、成人に対して本発明のオリゴマーの量として、1日当たり0.1mg～10g/ヒトの範囲内が、好ましくは1 mg～1 g/ヒトの範囲内が一般的である。この数値は標的とする疾患の種類、投与形態、標的分子によっても異なる場合がある。従って、場合によってはこれ以下でも十分であるし、また逆にこれ以上の用量を必要とするときもある。また1日1回から数回の投与又は1日から数日間の間隔で投与することができる。

【００９７】

本発明の組成物の別の態様として、本発明のオリゴヌクレオチドを発現し得るベクターと上述した担体とを含む医薬組成物を挙げることができる。かかる発現ベクターは、複数の本発明のオリゴヌクレオチドを発現し得るものであってもよい。当該組成物には、本発明のオリゴマーを含有する本発明の組成物と同様に、医薬的に許容可能な添加剤を添加することができる。当該組成物中に含まれる発現ベクターの濃度は、担体の種類等によって異なるが、0.1 nM～100 μMの範囲内が適当であり、1 nM～10 μMの範囲内が好ましく、10 nM～1 μMの範囲内がより好ましい。当該組成物中に含まれる発現ベクターと担体との重量比（担体/発現ベクター）は、発現ベクターの性質、担体の種類等によって異なるが、0.1～100の範囲内が適当であり、1～50の範囲内が好ましく、10～20の範囲内がより好ましい。また、当該組成物中に含まれる担体の含有量は、本発明のオリゴマーを含有する本発明の組成物の場合と同様であり、その調製方法等に関しても、本発明の組成物の場合と同様である。

【００９８】

以下に、実施例及び試験例を掲げて、本発明をさらに詳しく説明するが、本発明は実施例に示される範囲に限定されるものではない。

【実施例】

【００９９】

［参考例1］

<u>アミノメチルポリスチレン樹脂に担持された4－｛［（2S, 6R）－6－（4－ベンズアミド－2－オキソピリミジン－1－イル）－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸</u>

工程1：4－｛［（2S, 6R）－6－（4－ベンズアミド－2－オキソピリミジン－1（2H）－イル）－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸の製造

アルゴン雰囲気下、N－｛1－［（2R, 6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－2－オキソ－1, 2－ジヒドロピリミジン－4－イル｝ベンズアミド22.0gと4－ジメチルアミノピリジン（4－DMAP）7.04gをジクロロメタン269mLに懸濁し、無水コハク酸5.76gを加え、室温で3時間撹拌した。反応液にメタノール40mLを加え、減圧濃縮した。残渣に酢酸エチルと0.5Mのリン酸二水素カリウム水溶液を用いて抽出操作を行っ

た。得られた有機層を0.5Mのリン酸二水素カリウム水溶液、水、飽和食塩水の順で洗浄した。得られた有機層を硫酸ナトリウムで乾燥し、減圧濃縮し、25.9gの目的物を得た。

【０１００】

工程2：アミノメチルポリスチレン樹脂に担持された4－｛［（2S，6R）－6－（4－ベンズアミド－2－オキソピリミジン－1－イル）－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸の製造

4－｛［（2S，6R）－6－（4－ベンズアミド－2－オキソピリミジン－1（2H）－イル）－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸23.5gをピリジン（脱水）336mLに溶解し、4－DMAP4.28g、1－エチル－3－（3－ジメチルアミノプロピル）カルボジイミド塩酸塩40.3gを加えた。次いで、アミノメチルポリスチレン樹脂 1% DVB架橋（東京化成工業社製、A1543）25.0g、トリエチルアミン24mLを加え、室温で4日間振とうした。反応後、樹脂をろ取した。得られた樹脂をピリジン、メタノール、ジクロロメタンの順で洗浄し、減圧乾燥した。得られた樹脂にテトラヒドロフラン（脱水）150mL、無水酢酸15mL、2，6－ルチジン15mLを加え、室温で2時間振とうした。樹脂をろ取し、ピリジン、メタノール、ジクロロメタンの順で洗浄し、減圧乾燥し、33.7gの目的物を得た。

当該目的物のローディング量は、公知の方法を用いて、樹脂1g当たりのトリチルのモル量を409nmにおけるUV吸光度を測定することにより決定した。樹脂のローディング量は、397.4μmol/gであった。

【０１０１】

UV測定条件
機器：U-2910（日立製作所）
溶媒：メタンスルホン酸
波長：265 nm
ε値：45000

【０１０２】

［参考例2］

アミノメチルポリスチレン樹脂に担持された4－オキソ－4－｛［（2S，6R）－6－（6－オキソ－2－［2－フェノキシアセタミド］－1H－プリン－9－イル）－4－トリチルモルホリン－2－イル］メトキシ｝ブタン酸

工程1：N^2－（フェノキシアセチル）グアノシンの製造

グアノシン100gを80℃で減圧下、24時間乾燥した。ピリジン（脱水）500mL、ジクロロメタン（脱水）500mLを加え、アルゴン雰囲気下、0℃にてクロロトリメチルシラン401mLを滴下し室温で3時間撹拌した。再度氷冷し、フェノキシアセチルクロライド66.3gを滴下し、氷冷下、更に3時間撹拌した。反応液にメタノール500mlを加え、室温で終夜撹拌後、減圧下溶媒を留去した。残渣にメタノール500mLを加え、減圧下濃縮することを3回行った。残渣に水4Lを加え氷冷下1時間撹拌し、析出物をろ取した。このものを、水、次いで、冷メタノールで洗浄し、乾燥して目的化合物を150.2g得た（収率：102%）（参考：Org. Lett.（2004），Vol. 6，No. 15，2555－2557）。

【０１０３】

工程2：N－｛9－［（2R，6S）－6－（ヒドロキシメチル）－4－モルホリン－2－イル］－6－オキソ－6，9－ジヒドロ－1H－プリン－2－イル｝－2－フェノキシアセタミド p－トルエンスルホン酸塩

工程1で得られた化合物30gをメタノール480mLに懸濁し、氷冷下、2N塩酸130mLを加えた。次いで、四ほう酸アンモニウム4水和物56.8g、過ヨウ素酸ナトリウム16.2gをこの順で加え、室温で3時間撹拌した。反応液を氷冷し、不溶物をろ過して除き、これをメタノール100mLで洗浄した。ろ液と洗浄液を合わせて氷冷し、2－ピコリンボラン11.52gを加えて20分間撹拌後、p－トルエンスルホン酸・1水和物54.6gをゆっくり加えて、4℃で終夜撹拌した。析出物をろ取し、冷メタノール500mLで洗浄後、乾燥して目的化合物を17.7g得た（収率：43.3%）。

^{1}H NMR（δ，DMSO－d6）：9.9－9.2（2H，br）、8.35（1H，s）、7.55（2H，m）、7.35（

2H，m）、7.10（2H，d，J＝7.82Hz）、7.00（3H，m）、5.95（1H，dd， J＝10.64，2.42Hz）、4.85（2H，s）、4.00（1H，m）、3.90－3.60（2H，m）、3.50－3.20（5H，m）、2.90（1H，m）、2.25（3H，s）

【０１０４】

工程3：N－｛9－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－6－オキソ－6，9－ジヒドロ－1H－プリン－2－イル｝－2－フェノキシアセタミドの製造

工程2で得られた化合物2.0gをジクロロメタン30mLに懸濁し、氷冷下、トリエチルアミン13.9g、トリチルクロリド18.3gを加えて、室温で1時間撹拌した。反応液を飽和重曹水、次いで水で洗浄後乾燥し、有機層を減圧濃縮した。残渣に0.2Mクエン酸ナトリウム緩衝液（pH3）/メタノール（1：4（v/v））40mLを加えて撹拌し、次いで水40mLを加えて氷冷下1時間撹拌した。これをろ取し、冷メタノールで洗浄、乾燥して目的化合物を1.84g得た（収率：82.0%）。

【０１０５】

工程4：アミノメチルポリスチレン樹脂に担持された4－オキソ－4－｛［（2S，6R）－6－（6－オキソ－2－［2－フェノキシアセタミド］－1H－プリン－9－イル）－4－トリチルモルホリン－2－イル］メトキシ｝ブタン酸の製造

参考例1と同様の方法で標記化合物を製造した。但し、参考例1の工程1で用いたN－｛1－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－2－オキソ－1，2－ジヒドロピリミジン－4－イル｝ベンズアミドの代わりに、本工程では、N－｛9－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－6－オキソ－6，9－ジヒドロ－1H－プリン－2－イル｝－2－フェノキシアセタミドを使用した。

【０１０６】

［参考例3］

アミノメチルポリスチレン樹脂に担持された4－｛［（2S，6R）－6－(5－メチル－2，4－ジオキソ－3，4－ジヒドロピリミジン－1－イル)－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸

参考例1と同様の方法で標記化合物を製造した。但し、参考例1の工程1で用いたN－｛1－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－2－オキソ－1，2－ジヒドロピリミジン－4－イル｝ベンズアミドの代わりに、本工程では、1－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－5－メチルピリミジン－2，4（1H，3H）－ジオンを使用した。

【０１０７】

［参考例4］

アミノメチルポリスチレン樹脂に担持された1，12－ジオキソ－1－（4－トリチルピペラジン－1－イル）－2，5，8，11－テトラオキサ－15－ペンタデカン酸

参考例1と同様の方法で標記化合物を製造した。但し、参考例1の工程1で用いたN－｛1－［（2R，6S）－6－（ヒドロキシメチル）－4－トリチルモルホリン－2－イル］－2－オキソ－1，2－ジヒドロピリミジン－4－イル｝ベンズアミドの代わりに、本工程では、2－［2－（2－ヒドロキシエトキシ）エトキシ］エチル 4－トリチルピペラジン－1－カルボン酸（国際公開公報第2009/064471号に記載の化合物）を使用した。

【０１０８】

以下の実施例1～12、比較例1～3の記載に従い、表2 PMO No.1-11，13-16に示す各種PMOを合成した。合成したPMOを注射用水（大塚製薬工場社製）で溶解した。なお、PMO No.12はジーンツールズ社から購入した。

【表２】

表２

PMO No.	エクソン53中の標的配列	備考	配列番号
1	第31～55番目	5'末端：基(3)	配列番号4
2	第32～53番目	5'末端：基(3)	配列番号8
3	第32～56番目	5'末端：基(3)	配列番号11
4	第33～54番目	5'末端：基(3)	配列番号15
5	第34～58番目	5'末端：基(3)	配列番号25
6	第36～53番目	5'末端：基(3)	配列番号32
7	第36～55番目	5'末端：基(3)	配列番号34
8	第36～56番目	5'末端：基(3)	配列番号35
9	第36～57番目	5'末端：基(3)	配列番号36
10	第33～57番目	5'末端：基(3)	配列番号18
11	第39～69番目	非特許文献3のH53A(+39+69)（Table 1参照）に相当する配列 5'末端：基(3)	配列番号38
12	第30～59番目	非特許文献5のh53A30/1（Table 1参照）に相当する配列 5'末端：基(2)	配列番号39
13	第32～56番目	5'末端：基(1)	配列番号11
14	第36～56番目	5'末端：基(1)	配列番号35
15	第30～59番目	非特許文献5のh53A30/1（Table 1参照）に相当する配列 5'末端：基(3)	配列番号39
16	第23～47番目	特許文献4に記載のSEQ ID NO:429に相当する配列 5'末端：基(3)	配列番号47

【０１０９】

［実施例1］

PMO No. 8

アミノメチルポリスチレン樹脂に担持された4－｛［（2S，6R）－6－（4－ベンズアミド－2－オキソピリミジン－1（2H）－イル）－4－トリチルモルホリン－2－イル］メトキシ｝－4－オキソブタン酸（参考例1）2g（800μmol）を反応槽に移し入れ、ジクロロメタン30mLを添加し、30分間静置した。さらに、ジクロロメタン30mLで2回洗浄した後、下記合成サイクルを開始した。標記化合物の塩基配列になるよう、各サイクルにおいて所望のモルホリノモノマー化合物を添加した。

【表３】

表３

ステップ	試薬	量（mL）	時間（分）
1	デブロック溶液	30	2.0
2	デブロック溶液	30	2.0
3	デブロック溶液	30	2.0
4	デブロック溶液	30	2.0
5	デブロック溶液	30	2.0
6	デブロック溶液	30	2.0
7	中和溶液	30	1.5
8	中和溶液	30	1.5
9	中和溶液	30	1.5
10	中和溶液	30	1.5
11	中和溶液	30	1.5
12	中和溶液	30	1.5
13	ジクロロメタン	30	0.5
14	ジクロロメタン	30	0.5
15	ジクロロメタン	30	0.5
16	カップリング溶液Ｂ	20	0.5
17	カップリング溶液Ａ	6～11	90.0
18	ジクロロメタン	30	0.5
19	ジクロロメタン	30	0.5
20	ジクロロメタン	30	0.5
21	キャッピング溶液	30	3.0
22	キャッピング溶液	30	3.0
23	ジクロロメタン	30	0.5
24	ジクロロメタン	30	0.5
25	ジクロロメタン	30	0.5

【０１１０】

なお、デブロック溶液としては、トリフルオロ酢酸（2当量）とトリエチルアミン（1当量）の混合物を3%（w/v）になるように、1%（v/v）のエタノールと10%（v/v）の2，2，2−トリフルオロエタノールを含有するジクロロメタン溶液で溶解したものを用いた。

中和溶液としては、N，N－ジイソプロピルエチルアミンを5%（v/v）になるように、25%（v/v）の2－プロパノールを含有するジクロロメタン溶液で溶解したものを用いた。カップリング溶液Aとしては、モルホリノモノマー化合物を0.15Mになるように、10%（v/v）のN，N－ジイソプロピルエチルアミンを含有する1，3－ジメチル－2－イミダゾリジノンで溶解したものを用いた。カップリング溶液Bとしては、N，N－ジイソプロピルエチルアミンを10%（v/v）になるように、1，3－ジメチル－2－イミダゾリジノンで溶解したものを用いた。キャッピング溶液としては、ジクロロメタンに対して20%（v/v）の無水酢酸と30%（v/v）の2，6－ルチジンを溶解したものを使用した。

【０１１１】

上記で合成したPMOが担持されたアミノメチルポリスチレン樹脂を反応容器から回収し、2時間以上室温で減圧乾燥した。乾燥したアミノメチルポリスチレン樹脂に担持されたPMOを反応容器に入れ、28%アンモニア水－エタノール（1/4）200mLを加え、55℃で15時間撹拌した。アミノメチルポリスチレン樹脂をろ別し、水－エタノール（1/4）50mLで洗浄した。得られたろ液を減圧濃縮した。得られた残渣を20mMの酢酸－トリエチルアミン緩衝液（TEAA緩衝液）とアセトニトリルの混合溶媒（4/1）100mLに溶解し、メンブレンフィルターでろ過した。得られたろ液を逆相HPLCにて精製した。使用した条件は、以下の通りである。

【表４】

表4

カラム	XTerra　MS18 (Waters，φ50×100mm，1CV＝200mL)
流速	60mL/分
カラム温度	室温
A液	20mM　TEAA　緩衝液
B液	CH_3CN
Gradient	（B）　conc.　20→50%/9CV

各フラクションを分析して、アセトニトリル－水（1/1）100mLで目的物を回収し、エタノール200mLを添加し、減圧濃縮した。さらに、減圧乾燥し、白色固体を得た。得られた固体に10mMのリン酸水溶液300mLを加え、懸濁させた。2Mのリン酸水溶液10mLを加え、15分間撹拌した。さらに、2Mの水酸化ナトリウム水溶液15mLを加えて中和した。さらに、2Mの水酸化ナトリウム水溶液15mLを加えてアルカリ性とし、メンブレンフィルター（0.45μm）でろ過した。10mMの水酸化ナトリウム水溶液100mLで洗いこみ、水溶液として目的物を得た。

得られた目的物を含有する水溶液を陰イオン交換樹脂カラムで精製した。使用した条件は下記の通りである。

【表５】

表5

カラム	Source 30Q（GE Healthcare, φ40×150mm, 1CV=200mL）
流速	80 mL/min
カラム温度	室温
A液	10mMの水酸化ナトリウム水溶液
B液	10mMの水酸化ナトリウム水溶液，1Mの塩化ナトリウム水溶液
Gradient	（B） conc. 5→35%/15CV

各フラクションを分析（HPLC）し、目的物を水溶液として得た。得られた水溶液に0.1Mのリン酸緩衝液（pH 6.0）225mLを添加し中和した。メンブレンフィルター（0.45μm）でろ過した。次いで、下記条件で限外ろ過を行い脱塩した。

【表６】

表6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1m^2

【０１１２】

ろ液を濃縮し、約250mLの水溶液を得た。得られた水溶液をメンブレンフィルター（0.45μm）でろ過した。得られた水溶液を凍結乾燥して、白色綿状固体として1.5gの目的化合物を得た。

ESI－TOF－MS 計算値：6924.82

測定値：6923.54

【０１１３】

［実施例2］

PMO. No. 1

実施例1と同様の方法に従って、標記化合物を製造した。

MALDI－TOF－MS 計算値：8291.96

測定値：8296.24

【０１１４】

［実施例3］

PMO. No. 2

実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS 計算値：7310.13

測定値：7309.23

【０１１５】

［実施例4］

PMO. No. 3

実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS　計算値：8270.94
測定値：8270.55

【０１１６】

［実施例5］

PMO. No. 4

実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料として、アミノメチルポリスチレン樹脂に担持された4－（（（2S, 6R）－6－(5－メチル－2, 4－ジオキソ－3, 4－ジヒドロピリミジン－1（2H）－イル)－4－トリチルモルホリン－2－イル）メトキシ）－4－オキソブタン酸（参考例3）を使用した。

ESI－TOF－MS　計算値：7310.13
測定値：7310.17

【０１１７】

［実施例6］

PMO. No. 5

実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料として、アミノメチルポリスチレン樹脂に担持された4－（（（2S, 6R）－6－(5－メチル－2, 4－ジオキソ－3, 4－ジヒドロピリミジン－1（2H）－イル)－4－トリチルモルホリン－2－イル）メトキシ）－4－オキソブタン酸（参考例3）を使用した。

ESI－TOF－MS　計算値：8270.94
測定値：8270.20

【０１１８】

［実施例7］

PMO. No. 6

実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS　計算値：5964.01
測定値：5963.68

【０１１９】

［実施例8］

PMO. No. 7

実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS　計算値：6609.55
測定値：6608.85

【０１２０】

［実施例9］

PMO. No. 9

実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料として、アミノメチルポリスチレン樹脂に担持された4－オキソ－4－（（（2S, 6R）－6－（6－オキソ－2－（2－フェノキシアセタミド）－1H－プリン－9（6H）－イル）－4－トリチルモルホリン－2－イル）メトキシ）ブタン酸（参考例2）を使用した。

ESI－TOF－MS　計算値：7280.11
測定値：7279.42

【０１２１】

[実施例10]
PMO. No. 10
実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料として、アミノメチルポリスチレン樹脂に担持された4－オキソ－4－（（（2S, 6R）－6－（6－オキソ－2－（2－フェノキシアセタミド）－1H－プリン－9（6H）－イル）－4－トリチルモルホリン－2－イル）メトキシ）ブタン酸（参考例2）を使用した。

ESI－TOF－MS 計算値：8295.95
測定値：8295.91
【０１２２】
[実施例11]
PMO. No. 13
実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料としてアミノメチルポリスチレン樹脂に担持された1, 12－ジオキソ－1－（4－トリチルピペラジン－1－イル）－2, 5, 8, 11－テトラオキサ－15－ペンタデカン酸（参考例4）を使用した。

ESI－TOF－MS 計算値：7276.15
測定値：7276.69
【０１２３】
[実施例12]
PMO. No. 14
実施例1と同様の方法に従って、標記化合物を製造した。但し、出発原料としてアミノメチルポリスチレン樹脂に担持された1, 12－ジオキソ－1－（4－トリチルピペラジン－1－イル）－2, 5, 8, 11－テトラオキサ－15－ペンタデカン酸（参考例4）を使用した。

ESI－TOF－MS 計算値：8622.27
測定値：8622.29
【０１２４】
[比較例1]
PMO. No. 11
実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS 計算値：10274.63
測定値：10273.71
【０１２５】
[比較例2]
PMO. No. 15
実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS 計算値：9941.33
測定値：9940.77
【０１２６】
[比較例3]
PMO. No. 16
実施例1と同様の方法に従って、標記化合物を製造した。

ESI－TOF－MS 計算値：8238.94
測定値：8238.69
【０１２７】
[試験例1]

In vitroアッセイ

RD細胞（ヒト横紋筋肉腫細胞株）4×10^5個に対して、PMO No. 1～8の本発明のオリゴマー及びPMO No. 11のアンチセンスオリゴマー10 μMをAmaxa Cell Line Nucleofector Kit Lを用いてNucleofector II（Lonza）により導入した。プログラムはT-030を用いた。

【０１２８】

導入後、細胞を、10%ウシ胎児血清（FCS）（インビトロジェン社製）を含むEagle's minimal essential medium(EMEM)培地（シグマ社製、以下同じ） 2mL中、37℃、5% CO_2条件下で一晩培養した。細胞をPBS（ニッスイ社製、以下同じ）で2回洗浄した後、ISOGEN（ニッポンジーン社製）500 μl を細胞に添加し、数分間室温に放置して細胞を溶解させ、該溶解物をEppendorfチューブに回収した。ISOGENに添付のプロトコールに従ってtotal RNAを抽出した。抽出したtotal RNAの濃度はNanoDrop ND-1000（エル・エム・エス社製）を用いて測定した。

【０１２９】

抽出したtotal RNA 400 ngに対し、Titan One Tube RT-PCR Kit（ロシュ社製）を用いてOne-Step RT-PCRを行った。キットに添付のプロトコールに従って、反応液を調製した。サーマルサイクラーはPTC-100（MJ Research社製）を用いた。用いたRT-PCRのプログラムは、以下の通りである。

50℃、30分間：逆転写反応
94℃、2分間：熱変性
[94℃、10秒間；58℃、30秒間；68 ℃、45秒間]x 30サイクル：PCR増幅
68℃、7分間：ポリメラーゼの熱失活

【０１３０】

RT-PCRに使用したフォワードプライマーとリバースプライマーの塩基配列は以下の通りである。

フォワードプライマー：5'-AGGATTTGGAACAGAGGCGTC-3' （配列番号40）
リバースプライマー：5'-GTCTGCCACTGGCGGAGGTC-3' （配列番号41）

【０１３１】

次に、上記RT-PCRの増幅産物に対し、Taq DNA Polymerase（ロシュ社製）を用いてnested PCRを行った。用いたPCRプログラムは、以下の通りである。

94℃、2分間：熱変性
[94℃、15秒間；58℃、30秒間；68 ℃、45秒間]x 30サイクル：PCR増幅
68℃、7分間：ポリメラーゼの熱失活

【０１３２】

上記nested PCRに使用したフォワードプライマーとリバースプライマーの塩基配列は以下の通りである。

フォワードプライマー：5'-CATCAAGCAGAAGGCAACAA-3' （配列番号42）
リバースプライマー：5'-GAAGTTTCAGGGCCAAGTCA-3' （配列番号43）

【０１３３】

上記nested PCRの反応産物1 μlをBioanalyzer（アジレント社製）を用いて解析した。

エクソン53 がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定した。これら「A」及び「B」の測定値に基づき、以下の式に従って、スキッピング効率を求めた。

スキッピング効率（%）= A /（ A ＋ B ）x 100

【０１３４】

実験結果

結果を図1に示す。本実験により、PMO No. 1～8の本発明のオリゴマーは、いずれもPMO No. 11のアンチセンスオリゴマーと比べて、著しく高い効率でエクソン53をスキッピングさせることが判明した。特に、PMO No. 3及び8の本発明のオリゴマーは、PMO No. 11のアンチセンスオリゴマーと比べて、4倍以上高いエクソンスキッピング効率を示す。

【０１３５】

[試験例2]

ヒト線維芽細胞を用いたIn vitroアッセイ

ZsGreen1共発現レトロウイルスベクターによりTIG-119細胞（ヒト正常組織由来線維芽細胞、医薬基盤研究所）又は5017細胞（ヒトDMD患者由来線維芽細胞、Coriell Institute for Medical Research）にヒトmyoD遺伝子（配列番号44）を導入した。

4から5日間インキュベートした後に、FACSによりZsGreen陽性のMyoD転換線維芽細胞を回収し、5×10^4個/cm^2になるように12穴プレートに播種した。増殖培地は10% FCS及び1% Penicillin/Streptomycin（P/S）（シグマ　アルドリッチ社)を含むDulbecco's Modified Eagle Medium:Nutrient Mixture F-12（DMEM・F-12）(インビトロジェン社)を1 mL使用した。

24時間後に分化培地（2%ウマ血清(インビトロジェン社)、1% P/S及びITS Liquid Media Supplement（シグマ社）含有DMEM/F-12）に交換した。2、3日ごとに培地交換を行い12から14日間インキュベートし、筋管細胞に分化させた。

その後、分化培地を6 μMのEndo-Porter（ジーンツール社）含有分化培地に交換し、終濃度10μMになるようにモルホリノオリゴマーを添加した。48時間インキュベート後に、TRIzol （インビトロジェン社製）により、細胞からtotal RNAを抽出した。抽出したtotal RNA 50 ngに対し、QIAGEN OneStep RT-PCR Kitを用いてRT-PCRを行った。添付のプロトコールに従って、反応液を調製した。サーマルサイクラーはiCycler（Bio-Rad社製）を用いた。用いたRT-PCRのプログラムは、以下の通りである。

50℃、30分間：逆転写反応
95℃、15分間：熱変性
[94℃、1分間；60℃、1分間；72 ℃、1分間]x 35サイクル：PCR増幅
72℃、7分間：ポリメラーゼの熱失活

【０１３６】

プライマーはhEX51F及びhEX55Rを使用した。

hEX51F：5'-CGGGCTTGGACAGAACTTAC-3'　（配列番号45）
hEx55R：5'-TCCTTACGGGTAGCATCCTG-3'　（配列番号46）

【０１３７】

上記RT-PCR反応の反応産物を2%アガロースゲル電気泳動によって分離し、 GeneFlash（Syngene社）によりゲル写真を撮影した。Image J（アメリカ国立衛生研究所製）により、エクソン53 がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定した。これら「A」及び「B」の測定値に基づき、以下の式に従って、スキッピング効率を求めた。

スキッピング効率（%）= A /（ A ＋ B ）x 100

【０１３８】

実験結果

結果を図2及び図3に示す。本実験により、PMO No. 3、8及び9の本発明のオリゴマー（図2）は、TIG-119細胞において、いずれもPMO No. 12のアンチセンスオリゴマーと比べて、高い効率でエクソン53をスキッピングさせることが判明した（図2）。特に、PMO No. 3

及び8の本発明のオリゴマーは、PMO No. 12のアンチセンスオリゴマーと比べて、2倍以上高いエクソンスキッピング効率を示す（図2）。

また、本実験により、PMO No. 3及び8～10の本発明のオリゴマー（図3）は、5017細胞において、いずれもPMO No. 12のアンチセンスオリゴマーと比べて、高い効率でエクソン53をスキッピングさせることが判明した（図3）。特に、PMO No. 3及び8の本発明のオリゴマーは、PMO No. 12のアンチセンスオリゴマーと比べて、7倍以上高いエクソンスキッピング効率を示す（図3）。

【０１３９】

[試験例3]

ヒト線維芽細胞を用いたIn vitroアッセイ

エクソン45から52が欠失したDMD患者またはエクソン48から52が欠失したDMD患者の左上腕内側より生検し、皮膚線維芽細胞株（ヒトDMD患者（エクソン45-52またはエクソン48-52）由来線維芽細胞）を樹立した。ZsGreen1共発現レトロウイルスベクターにより線維芽細胞にヒトmyoD遺伝子（配列番号44）を導入した。

4から5日間インキュベートした後に、FACSによりZsGreen陽性のMyoD転換線維芽細胞を回収し、5×10^4個/cm^2になるように12穴プレートに播種した。増殖培地は10%FCS及び1%Penicillin/Streptomycin（P/S）（シグマ　アルドリッチ社)を含むDulbecco's Modified Eagle Medium: Nutrient Mixture F-12（DMEM/F-12）(インビトロジェン社)を1 mL使用した。

24時間後に分化培地（2%ウマ血清(インビトロジェン社)、1%P/S及びITS Liquid Media Supplement（シグマ社）含有DMEM/F-12）に交換した。2、3日ごとに培地交換を行い12、14または20日間インキュベートし、筋管細胞に分化させた。

その後、分化培地を6 μMのEndo-Porter（ジーンツール社）含有分化培地に交換し、終濃度10μMになるようにモルホリノオリゴマーを添加した。48時間インキュベート後に、TRIzol （インビトロジェン社製）により、細胞からtotal RNAを抽出した。抽出したtotal RNA 50 ngに対し、QIAGEN OneStep RT-PCR Kitを用いてRT-PCRを行った。添付のプロトコールに従って、反応液を調製した。サーマルサイクラーはiCycler（Bio-Rad社製）を用いた。用いたRT-PCRのプログラムは、以下の通りである。

50℃、30分間：逆転写反応
95℃、15分間：熱変性
[94℃、1分間；60℃、1分間；72 ℃、1分間]x 35サイクル：PCR増幅
72℃、7分間：ポリメラーゼの熱失活

【０１４０】

プライマーはhEx44F及びh55Rを使用した。

hEx44F：5’ - TGTTGAGAAATGGCGGCGT-3’　（配列番号48）
hEx55R：5’ - TCCTTACGGGTAGCATCCTG-3’　（配列番号46）

【０１４１】

上記RT-PCR反応の反応産物を2%アガロースゲル電気泳動によって分離し、 GeneFlash（Syngene社）によりゲル写真を撮影した。Image J（アメリカ国立衛生研究所製）により、エクソン53 がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定した。これら「A」及び「B」の測定値に基づき、以下の式に従って、スキッピング効率を求めた。

スキッピング効率（%）= A /（ A ＋ B ）x 100

【０１４２】

実験結果

結果を図4及び図5に示す。本実験により、PMO No. 3及び8の本発明のオリゴマーは、エ

クソン45-52欠失（図4）またはエクソン48-52欠失（図5）DMD患者由来細胞において、80%以上の高い効率でエクソン53をスキッピングさせることが判明した。また、PMO No. 3及び8の本発明のオリゴマーは、エクソン45-52欠失DMD患者由来細胞において、PMO No.15のアンチセンスオリゴマーと比較して高い効率でエクソン53をスキッピングさせることが判明した（図4）。

【０１４３】

[試験例4]

ウェスタンブロッティング

PMO No.8の本発明のオリゴマーを10μMの濃度で細胞に添加し、72時間後の細胞からComplete Mini (Roche Applied Science社製)含有RIPA buffer (Thermo Fisher Scientific社製）でタンパク質を抽出し、BCA protein assay kit (Thermo Fisher Scientific社製)でタンパク質を定量した。NuPAGE Novex Tris-Acetate Gel 3-8% (Invitrogen社製）で150 V、75分間電気泳動、セミドライブロッターでPVDF膜 (Millipore社製)へ転写した。PVDF膜を5% ECL Blocking agent(GE Healthcare社製）でブロッキング後、抗ジストロフィン抗体 (NCL-Dys1, Novocastra社製)溶液中で膜をインキュベートした。さらに、peroxidase-conjugated goat-antimouse IgG (型番、Bio-Rad)溶液中でインキュベートした後、 ECL Plus Western blotting system (GE Healthcare社製)により発色した。

【０１４４】

免疫染色

PMO No.3またはNo.8の本発明のオリゴマーを細胞に添加し、72時間後の細胞を3% paraformaldehyde、10分間で固定化した。10% Triton-Xで10分間インキュベートした。10%ヤギ血清含有PBSでブロッキング、抗ジストロフィン抗体(NCL-Dys1, Novocastra)溶液中で膜をインキュベート、さらに抗マウスIgG抗体(Invitrogen社製)溶液中で膜をインキュベートした。Pro Long Gold Antifade reagent (Invitrogen社製)でマウントし、蛍光顕微鏡で観察した。

【０１４５】

実験結果

結果を図6及び図7に示す。本実験により、PMO No.3及び8の本発明のオリゴマーはジストロフィンタンパク質の発現を誘導することがウェスタンブロッティング（図6）及び免疫染色（図7）により確認できた。

【０１４６】

[試験例5]

ヒト線維芽細胞を用いたIn vitroアッセイ

試験例3と同様の方法で実験を行った。

【０１４７】

実験結果

結果を図8に示す。本実験により、PMO No. 3及び8の本発明のオリゴマーは、エクソン45-52欠失DMD患者由来細胞において、PMO No. 13及び14の本発明のオリゴマーよりも高い効率でエクソン53をスキッピングさせることが判明した（図8）。

【０１４８】

[試験例6]

In vitroアッセイ

配列番号49～123に記載の2’-O-メトキシ-ホスホロチオエート体（2’-OMe-S-RNA）のアンチセンスオリゴマーを用いて実験を行った。アッセイに用いた各種アンチセンスオリゴマーは日本バイオサービス社より購入した。各種アンチセンスオリゴマーの配列を以下に示す。

【表７－Ａ】

表７

アンチセンスオリゴマー	塩基配列	配列番号
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73

【表７－Ｂ】

H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101

【表７－Ｃ】

H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

【０１４９】

RD細胞（ヒト横紋筋肉腫細胞株）3×10^5個を6穴プレートに播種し、10%ウシ胎児血清（FCS）（インビトロジェン社製）を含むEagle's minimal essential medium(EMEM)培地（シグマ社製、以下同じ）2mL中、37℃、5%CO_2条件下で一晩培養した。上記のエクソン53スキッピング用の各種アンチセンスオリゴマー（日本バイオサービス社製）（1 μM）とLipofectamine2000（インビトロジェン社製）の複合体を作成し、1.8mLで培地交換したRD細胞に、200μl添加し、終濃度100 nMとした。

添加後、一晩培養した。細胞をPBS（ニッスイ社製、以下同じ）で2回洗浄した後、ISOGEN（ニッポンジーン社製）500 μl を細胞に添加し、数分間室温に放置して細胞を溶解させ、該溶解物をEppendorfチューブに回収した。ISOGENに添付のプロトコールに従ってtotal RNAを抽出した。抽出したtotal RNAの濃度はNanoDrop ND-1000（エル・エム・エス

社製）を用いて測定した。
抽出したtotal RNA 400 ngに対し、Titan One Tube RT-PCR Kit（ロシュ社製）を用いてOne-Step RT-PCRを行った。キットに添付のプロトコールに従って、反応液を調製した。サーマルサイクラーはPTC-100（MJ Research社製）を用いた。用いたRT-PCRのプログラムは、以下の通りである。

50℃、30分間：逆転写反応
94℃、2分間：熱変性
[94℃、10秒間；58℃、30秒間；68 ℃、45秒間]x 30サイクル：PCR増幅
68℃、7分間：ポリメラーゼの熱失活

【０１５０】
RT-PCRに使用したフォワードプライマーとリバースプライマーの塩基配列は以下の通りである。

フォワードプライマー：5’-CATCAAGCAGAAGGCAACAA-3’ （配列番号42）
リバースプライマー：5’-GAAGTTTCAGGGCCAAGTCA-3’ （配列番号43）

次に、上記RT-PCRの増幅産物に対し、Taq DNA Polymerase（ロシュ社製）を用いてnested PCRを行った。用いたPCRプログラムは、以下の通りである。

94℃、2分間：熱変性
[94℃、15秒間；58℃、30秒間；68 ℃、45秒間]x 30サイクル：PCR増幅
68℃、7分間：ポリメラーゼの熱失活

【０１５１】
上記nested PCRに使用したフォワードプライマーとリバースプライマーの塩基配列は以下の通りである。

フォワードプライマー：5’-AGGATTTGGAACAGAGGCGTC-3’ （配列番号40）
リバースプライマー：5’-GTCTGCCACTGGCGGAGGTC-3’ （配列番号41）

【０１５２】
上記nested PCRの反応産物1 μlをBioanalyzer（アジレント社製）を用いて解析した。
エクソン53 がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定した。これら「A」及び「B」の測定値に基づき、以下の式に従って、スキッピング効率を求めた。

スキッピング効率（％）＝ A／（ A ＋ B ）x 100

【０１５３】
実験結果
結果を図9から図17に示す。本実験により、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第31～61番目にアンチセンスオリゴマーを設計した場合、高い効率でエクソン53をスキッピングさせることが判明した。
【０１５４】
[試験例7]
RD細胞（ヒト横紋筋肉腫細胞株）3.5× 10^5個に対して、アンチセンスオリゴマー0.3～30 μMをAmaxa Cell Line Nucleofector Kit Lを用いてNucleofector II（Lonza）により導入した。プログラムはT-030を用いた。

導入後、細胞を、10％ウシ胎児血清（FCS）（インビトロジェン社製）を含むEagle's minimal essential medium(EMEM)培地（シグマ社製、以下同じ） 2mL中、37℃、5％CO_2条件下で一晩培養した。細胞をPBS（ニッスイ社製、以下同じ）で2回洗浄した後、ISOGEN（ニッポンジーン社製）500 μl を細胞に添加し、数分間室温に放置して細胞を溶解させ、該溶解物をEppendorfチューブに回収した。ISOGENに添付のプロトコールに従ってtotal RNAを抽出した。抽出したtotal RNAの濃度はNanoDrop ND-1000（エル・エム・エス社製）を用いて測定した。

抽出したtotal RNA 400 ngに対し、QIAGEN OneStep RT-PCR Kit（キアゲン社製）を用いてOne-Step RT-PCRを行った。キットに添付のプロトコールに従って、反応液を調製した。サーマルサイクラーはPTC-100（MJ Research社製）を用いた。用いたRT-PCRのプログラムは、以下の通りである。

50℃、30分間：逆転写反応
95℃、15分間：熱変性
[94℃、30秒間；60℃、30秒間；72 ℃、1分間]x 35サイクル：PCR増幅
72℃、10分間：ポリメラーゼの熱失活

【０１５５】
RT-PCRに使用したフォワードプライマーとリバースプライマーの塩基配列は以下の通りである。

フォワードプライマー：5'-CATCAAGCAGAAGGCAACAA-3' （配列番号42）
リバースプライマー：5'-GAAGTTTCAGGGCCAAGTCA-3' （配列番号43）

【０１５６】
上記PCRの反応産物1 μlをBioanalyzer（アジレント社製）を用いて解析した。

エクソン53 がスキップしたバンドのポリヌクレオチド量「A」と、エクソン53がスキップしなかったバンドのポリヌクレオチド量「B」を測定した。これら「A」及び「B」の測定値に基づき、以下の式に従って、スキッピング効率を求めた。

スキッピング効率（％）＝ A /（ A ＋ B ）x 100

【０１５７】
実験結果

結果を図18及び19に示す。本実験により、PMO No.8の本発明のオリゴマーは、PMO No.15及び16のアンチセンスオリゴマーと比べて、著しく高い効率でエクソン53をスキッピングさせることが判明した(図18)。また、PMO No. 3及び8の本発明のオリゴマーは、PMO No. 13及び14の本発明のオリゴマーよりも著しく高い効率でエクソン53をスキッピングさせることが判明した(図19)。この結果から、同一配列であっても、5'末端が-OH基である方が、スキッピング効率が高いことが示される。

【産業上の利用可能性】

【０１５８】
試験例に示す実験結果から、本発明のオリゴマー（PMO No. 1～10）は、従来技術に係るオリゴマー（PMO No. 11、12、15及び16）と比べ、いずれの細胞環境においても、著しく高い効率でエクソン53をスキッピングさせることが示された。また、試験例2で用いた5017細胞は、DMD患者から採取した細胞であり、さらに、試験例3及び5で用いた線維芽細胞もDMD患者由来のエクソン53スキッピング対象細胞である。特に、試験例3及び5において、本発明のオリゴマーは、エクソン53スキッピング対象となるDMD患者由来の細胞において、90％以上のエクソン53スキッピング効率を示すため、本発明のオリゴマーは、実際にDMD患者に投与した場合にも、高効率にエクソン53をスキッピングさせるといえる。

従って、本発明のオリゴマーは、DMDの治療において、非常に有用である。

【配列表フリーテキスト】
【０１５９】
配列番号2：合成核酸
配列番号3：合成核酸
配列番号4：合成核酸
配列番号5：合成核酸
配列番号6：合成核酸
配列番号7：合成核酸
配列番号8：合成核酸
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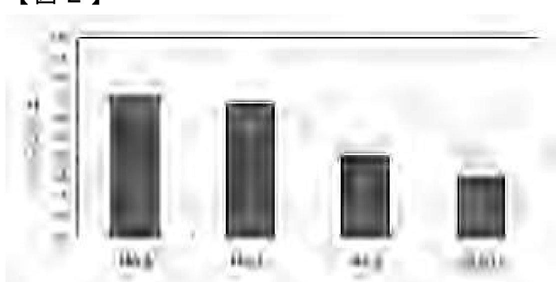
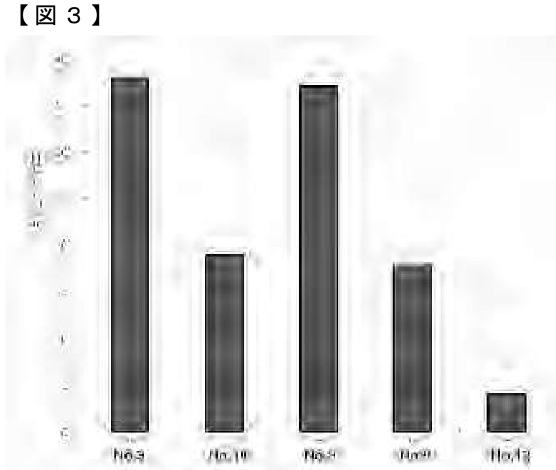
配列番号123：合成核酸

【図１】

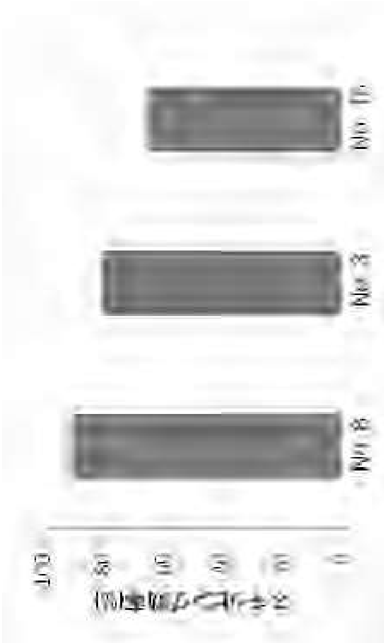
【図２】



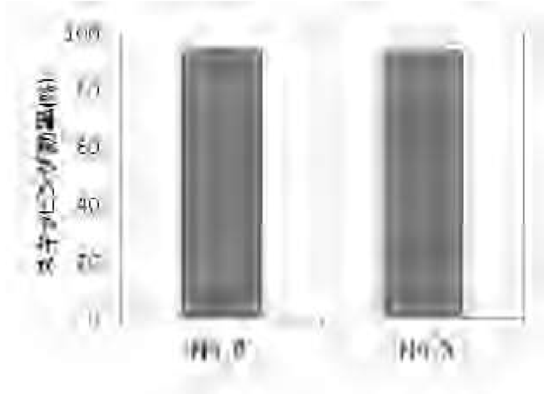
【図３】



【図４】

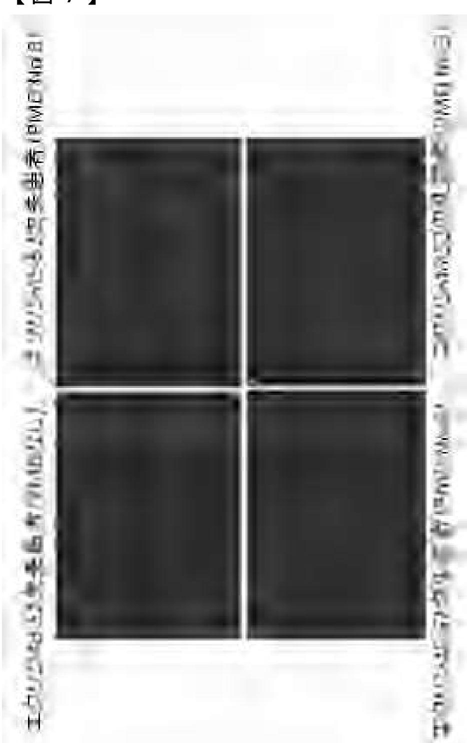


【図５】

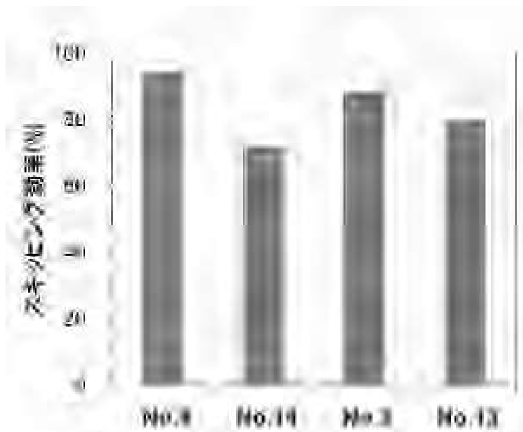


【図６】

【図７】



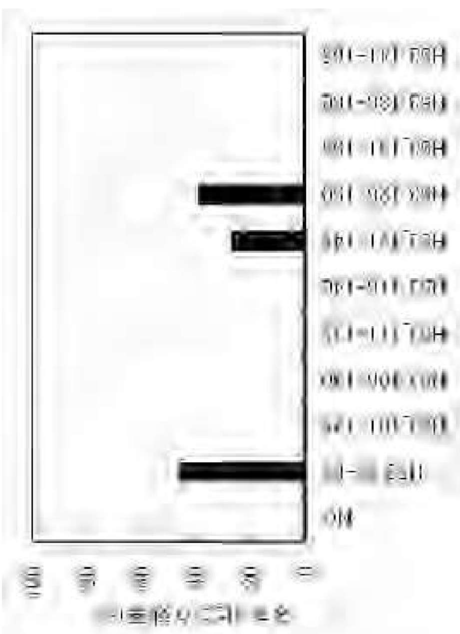
【図８】



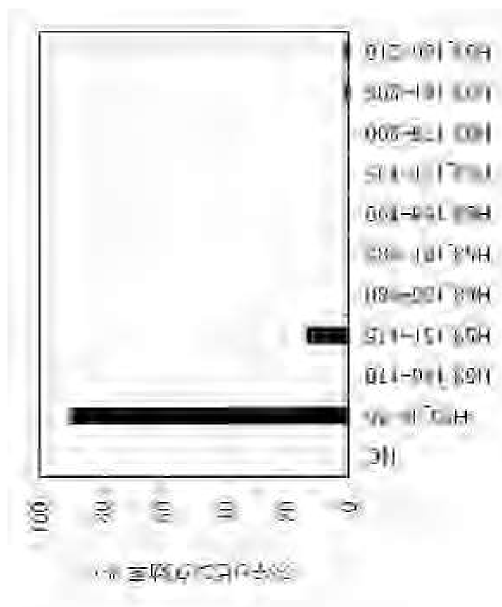
【図９】

【図１０】

【図１１】

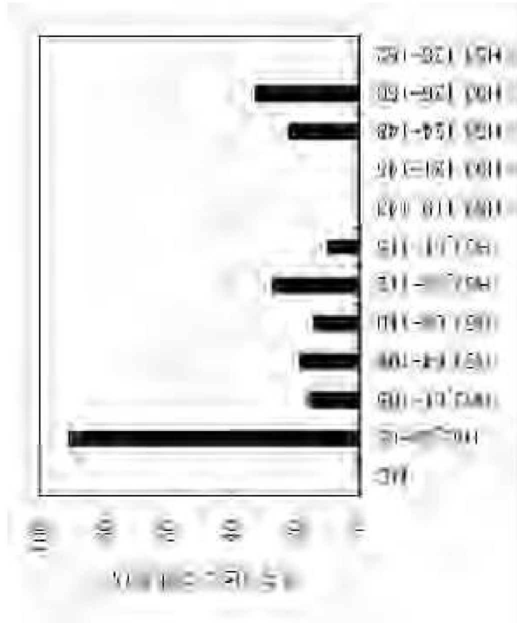


【図１２】



【図１３】

【図１４】

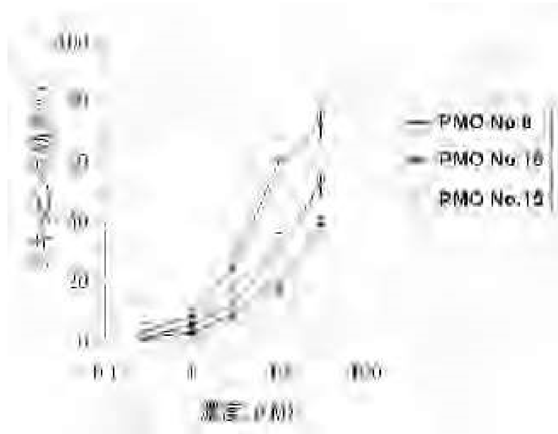


【図１５】

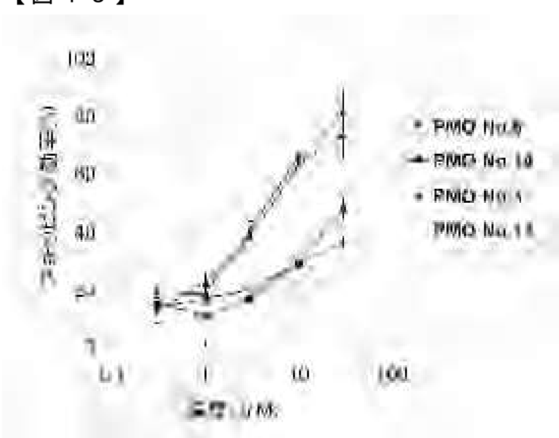
【図１６】

【図１７】

【図１８】



【図１９】



【配列表】
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米国特許出願公開第２０１０／０１６８２１２（ＵＳ，Ａ１）
特開２００２－０１０７９０（ＪＰ，Ａ）
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(58)調査した分野(Int.Cl., ＤＢ名)
Ｃ１２Ｎ １／００－１５／９０
ＣＡ／ＭＥＤＬＩＮＥ／ＢＩＯＳＩＳ／ＷＰＩＤＳ（ＳＴＮ）
ＪＳＴＰｌｕｓ／ＪＭＥＤＰｌｕｓ／ＪＳＴ７５８０（ＪＤｒｅａｍＩＩＩ）
Ｇｅｎｂａｎｋ／ＥＭＢＬ／ＤＤＢＪ／ＧｅｎｅＳｅｑ
ＰｕｂＭｅｄ

EXHIBIT AR

【書類名】 意見書
【整理番号】 P12-0190-B
【提出日】 平成29年 3月 9日
【あて先】 特許庁審査官 福澤 洋光 殿
【事件の表示】
　【出願番号】 特願2015-256962
【特許出願人】
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　【電話番号】 03-3273-2611
　【連絡先】 担当弁理士 今里 崇之
【発送番号】 514123
【意見の内容】

1．拒絶理由

　本件特許出願（以下「本願」という）に対する拒絶理由通知（以下「本拒絶理由通知」という）において、

　本願請求項1～10に係る発明は、特許法第39条第2項の規定により、特許を受けることができない（理由1：同日出願）、

　本願請求項1～10に係る発明は、特許法第29条第2項の規定により、特許を受けることができない（理由2：進歩性欠如）、

　と指摘されています。

2．補正

　上記拒絶理由に鑑み、出願人は特許請求の範囲及び明細書を以下のとおり補正しました。

（2-1）

　請求項1に記載のアンチセンスオリゴマーを「ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第36～60番目のヌクレオチドからなる配列に相補的な塩基配列からなる」ものに限定しました。

　当該補正はマーカッシュ形式で提示された選択肢を削除するものです。

（2-2）

　請求項9に記載のアンチセンスオリゴマーを「配列番号57に示す塩基配列からなる」ものに限定しました。

　当該補正はマーカッシュ形式で提示された選択肢を削除するものです。

（2-3）

　上記補正は、いずれも新規事項を追加するものではなく補正の要件を満たします（特許法第17条の2第3項）。

3．意見

　以下、補正後の本発明について意見を申し述べます。

（3-1） 理由1

　本願の請求項1は以下のとおりです。

「ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマーであって、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第36～60番目のヌクレオチドからなる配列に相補的な塩基配列からなる、アンチセンスオリゴマー」

これに対し出願1の請求項1に係るアンチセンスオリゴマーは「ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第33～54番目、第34～58番目、第36～55番目、第32～53番目、第36～53番目又は第36～57番目のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列からなる、アンチセンスオリゴマー」です。

両者を対比すると、本願請求項1に係るアンチセンスオリゴマーはその配列がヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第36～60番目のヌクレオチドからなる配列に相補的な塩基配列からなるのに対し、出願1の請求項1に係るアンチセンスオリゴマーは同エクソンの5’末端から第33～54番目、第34～58番目、第36～55番目、第32～53番目、第36～53番目又は第36～57番目のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列からなる点で両者は発明特定事項が相違します。
本願及び出願1の他の請求項は全て請求項1を引用しますので、本願及び出願1は他の請求項においても発明が相違します。

したがって、理由1は解消しました。

(3-2)　理由2
本願の請求項1は以下のとおりです。
「ヒトジストロフィン遺伝子の第53番目のエクソンのスキッピングを可能にするアンチセンスオリゴマーであって、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第36～60番目のヌクレオチドからなる配列に相補的な塩基配列からなる、アンチセンスオリゴマー。」
以下、簡略化のため請求項1に係るオリゴマーを「本発明のオリゴマー」と称し、本発明のオリゴマーの配列を「第36～60番目」と称します。
本発明は、ジストロフィン遺伝子のエクソン53のスキッピングを強く誘導するアンチセンスオリゴマーを提供するという課題（本願明細書の段落0011）を解決することを目的として発明されたものです。

これに対し引用文献2には、ヒトジストロフィン遺伝子の第53番目のエクソンの5’末端から第38～62番目(h53AON1)及び第23～47番目(H53A(+23+47))のヌクレオチドからなる配列のいずれか1つに相補的な塩基配列からなるアンチセンスオリゴマーが記載されています（Example 3）。

本発明と引用文献2に記載の発明とを対比すると本発明のオリゴマーは配列が第36～60番目に特定されていますが、引用文献2には当該配列を有するオリゴマーが記載されていない点で両者は相違します。
以下に、当該相違点に係る構成は容易に想到し得ないこと、及び本発明が当該相違点に係る構成により優れた効果を奏することをご説明致します。

（i）相違点に係る構成は容易に想到し得ないこと
引用文献2の実施例3ではエクソン53のスキッピング活性を検証し、そのうちSEQ ID NO: 429のオリゴマーは図4B-Fに示すとおり、最も高効率にエクソン53スキッピングを促したと記載されています。そして、SEQ ID NO: 429のオリゴマーがH53A(+23+47)（SEQ ID NO: 609）と同一の活性を有することが証明されたと記載されています。
この記載からH53A(+23+47)（SEQ ID NO: 609）は最も高いスキッピング活性（と少なく

とも同等の活性）を有することが分かります。
　また、図4H（下図）からも、H53A(+23+47)（SEQ ID NO: 609）とSEQ ID NO: 429のオリゴマーのみが効率的にスキッピングを誘導しているのに対し、他のオリゴマー（H53A(+39+69)、H53AON1、H53A(-12+10)、008）はスキッピング活性において劣っており、ほとんどスキッピングが生じていないことが分かります。

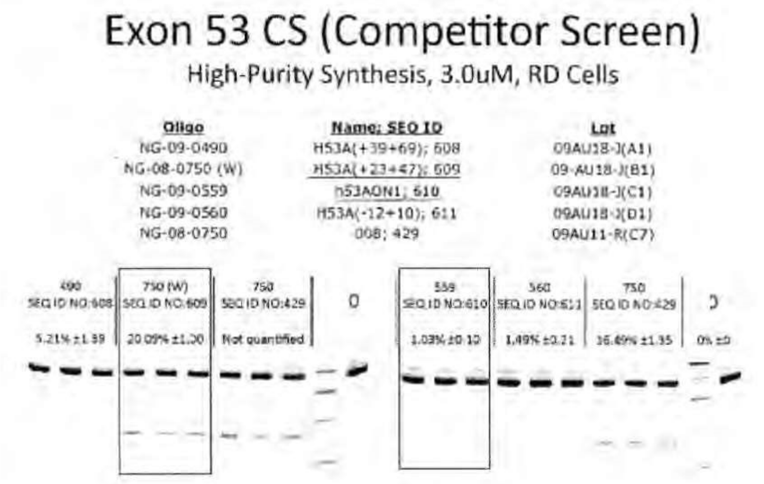
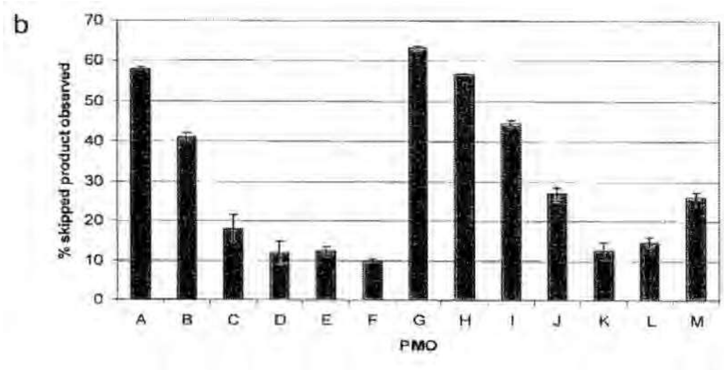


FIG. 4H

　したがって、本発明の課題の解決を目的とする当業者であれば、引用文献2の記載に接した際に当然にH53A(+23+47)に着目したはずです。
　そして、スキッピングを強く誘導するアンチセンスオリゴマーを得るために、第23～47番目の配列又はその周辺をターゲットするオリゴマーを作製することを動機付けられたはずです。
　一方、引用文献2に開示されるオリゴマーのうち本発明のオリゴマー（第36～60番目）に近い配列を有するのはh53AON1（第38～62番目）ですが、これは引用文献2の実施例3の記載や図4Hのデータを見てもスキッピング効率が低いことが明らかです。この点から、第38～62番目の配列やその周辺は、スキッピングのターゲットとして当業者の興味を惹くことはなかったはずです。

　したがって、引用文献2の記載を総合的に判断すれば、引用文献2には当業者が相違点に係る構成を想到することについて明確な反対動機があることが分かります。

　次に、引用文献3の記載を検証すると、引用文献3ではPMO-G（第30～59番目）が最も高い活性を有することが示され、一方で本発明のオリゴマー（第36～60番目）に近い配列を有するPMO-A（第35～59番目：本発明のオリゴマーの配列から塩基配列が1塩基ずれたもの）はPMO-Gよりも活性が低いことが示されています（図8b：下図）。
　それゆえ、引用文献2の場合と同様、引用文献3の記載に接した当業者は高い活性を求めて第30～59番目をターゲットすることを動機付けられるものといえます。



したがって、当業者は、引用文献2及び3の記載に接したとしても、本発明のオリゴマーのターゲット配列を探索することを動機付けられるはずはなく、その結果として相違点に係る構成を容易に想到することはできません。

以上より、相違点に係る構成は引用文献の記載から容易に想到し得るものではありませんので、本発明は進歩性を有します。

（ii）本発明が当該相違点に係る構成により優れた効果を奏すること

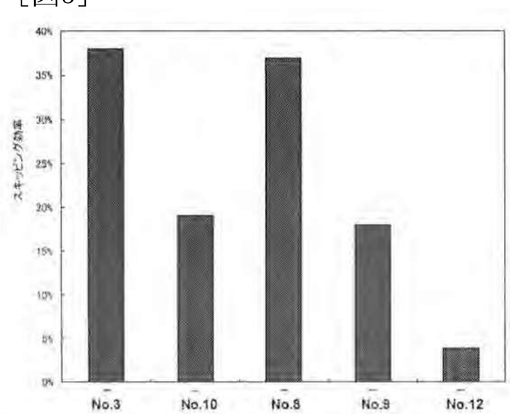
本発明のオリゴマーは、引用文献2及び3に記載のオリゴマーと比較して優れたスキッピング活性を有することが実験により確認されています。

引用文献2において最もスキッピング活性が高いとされるSEQ ID NO：429のオリゴマーと、引用文献3で最もスキッピング活性が高いとされるPMO-G（第30～59番目）はそれぞれ本願実施例においてPMO No. 16及びPMO No. 12として作製されています。

まず引用文献3のオリゴマーとの比較についてご説明します。

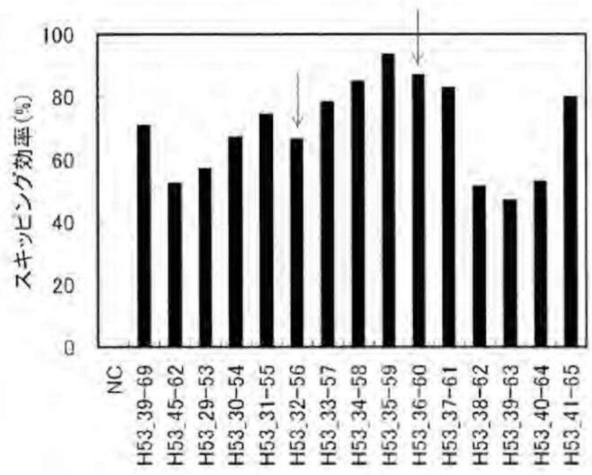
第一に、本願の図3から、PMO No. 12よりもPMO No. 3（第32～56番目）の方が活性が高いことが分かります。

［図3］



次に、本願の図16を参照すると、本発明のオリゴマーに相当するH53_36-60は、PMO No. 3に相当するH53_32-56よりも高い活性を有することが分かります。

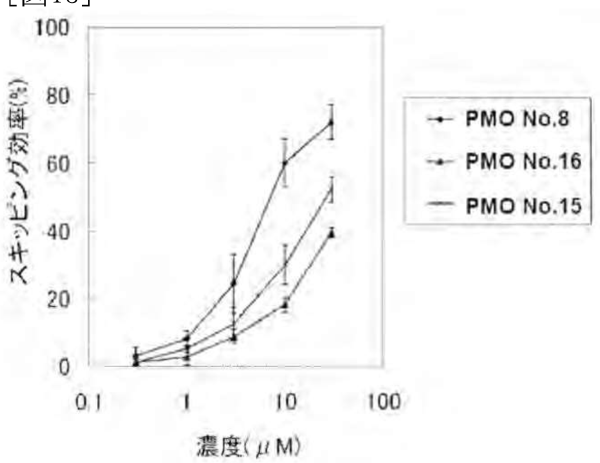
［図16］



したがって、本発明のオリゴマーとPMO NO. 3と引用文献3のPMO-G（第30～59番目）は活性の高さが、「本発明のオリゴマー＞PMO No. 3＞引用文献3のPMO-G」となっており、本発明のオリゴマーが引用文献3のPMO-Gよりも活性が高いことが分かります。

一方、引用文献2のオリゴマーとの比較については、本願図18から、引用文献2のオリゴマーに相当するPMO No. 16よりもPMO No. 8（第36～56番目）の方が活性が高いことが分かります。

［図18］



次に、図2～4からPMO No. 8はPMO No. 3と同程度の活性を有することが分かります。上で述べたとおり、本発明のオリゴマーはPMO No. 3よりも活性が高いので、本発明のオリゴマー、PMO No. 8、PMO NO. 3と引用文献2のH53A(+23+47)（PMO No. 16）は活性の高さが、「本発明のオリゴマー＞PMO No. 3≒PMO No. 8＞引用文献2のH53A(+23+47)」となっており、本発明のオリゴマーが引用文献2のH53A(+23+47)よりも活性が高いことが分かります。

引用文献2及び3では最もスキッピング活性が高いオリゴマーは、本発明のオリゴマーのターゲット配列（第36～60番目）とは異なる領域をターゲットすることが示されていますので、本発明のオリゴマーが引用文献2及び3のオリゴマーを上回る活性を有することはこれらの文献の記載からは予測し得ない優れた効果であるといえます。

以上より、本発明のオリゴマーは、引用文献の記載から当業者が予測し得ない優れた効果を奏するので、効果の面からも進歩性を有します。

（iii）小括

上で述べたとおり、本発明のオリゴマーは、引用文献の記載からその構成が容易に想到できるものではなく、かつ予測し得ない優れた効果を奏するので進歩性を有します。また、請求項2～10は全て請求項1を引用しますので、これらの請求項に係る発明も同様に進歩性を有します。

4．まとめ

以上のことから、拒絶理由は全て解消したものと思料致しますので、本願につき再度ご審査の上、特許すべき旨の査定を賜りますようお願い申し上げます。

以上

[Document Name]Written Opinion
[Reference Number]P12-0190-B
[Date of submission]Heisei 29(2017) March 9
[Recipient] Patent examiner FUKUZAWA, Hiromitsu
[Indication of case]
[Application number]Japanese Patent Application No. 2015-256962
[Patent applicant]
[Identification Number]000004156
[Name]Nippon Shinyaku Co.,Ltd.
[Patent applicant]
[Identification Number]510147776
[Name]National Research and Development Agency National Center of Neurology and Psychiatry
[Representative]
[Identification Number]100092783
[Patent attorney]
[Name]KOBAYASHI, Hiroshi
[Tel No.]03-3273-2611
[Contact information]The patent attorney in charge IMAZATO, Takayuki
[Dispatch number] 514123
[The contents of the opinion] 1. Reason for Refusal [in Notification of Reasons for Refusal (henceforth "this Notification of Reasons for Refusal") to Patent Application (henceforth "this Application")] [Invention Concerning Claim 1 in this Application - 10] invention which relates to the claim 1 in this application - 10 which cannot obtain a patent (Reason 1: Applications Filed on the Same Date) in accordance with the provisions of Article 39(2) of the Patent Act -- in accordance with the provisions of Article 29(2) of the Patent Act -- a patent -- it cannot receive (Reason 2: lack of inventive step) -- it is pointed out.

2. In view of the correction above-mentioned reason for refusal, the applicant corrected the scope of the claim and the Description as follows.
(2-1)
The antisense oligomer according to claim 1 was limited to" thing which consists of a base sequence complementary to the arrangement which consists of the 36-60th nucleotides from the five prime end of the 53rd exon of "Homo sapiens dystrophin gene. The correction concerned deletes the choice shown by the Markush form.
(2-2)
The antisense oligomer according to claim 9 was limited to" thing which consists of the base sequence shown in "SEQ ID NO 57. The correction concerned deletes the choice shown by the Markush form.
(2-3)
No above-mentioned correction adds a New Matter, and correction complies with requirements it (Patent Law Article 17bis(3)).

3. Below an opinion states an opinion about the after Amendment present invention.

(3-1) Claim 1 of Reason 1 this application is as follows. "It is antisense oligomer which makes possible skipping of the 53rd exon of the Homo sapiens dystrophin gene, Antisense oligomer which consists of a base sequence complementary to the arrangement which consists of the 36-60th nucleotides from the five prime end of the 53rd exon of the Homo sapiens dystrophin gene"

"On the other hand, antisense oligomer concerning Claim 1 of the application 1 It is the 33-54th from the five prime end of the 53rd exon of the Homo sapiens dystrophin gene It is antisense oligomer which consists of a base sequence of arrangement complementary to any one which consists of the 34-58th, the 36-55th, the 32-53rd, the 36-53rd, or the 36-57th nucleotides."

If both are contrasted, while the arrangement consists of a base sequence complementary to the arrangement which consists of the 36-60th nucleotides from the five prime end of the 53rd exon of the Homo sapiens dystrophin gene, [antisense oligomer concerning claim 1 in this application] Antisense oligomer concerning Claim 1 of the application 1 is the 33-54th from the five prime end of the exon As for both, a matters specifying the invention is different at the point which consists of the base sequence of arrangement complementary to any one which consists of the 34-58th, the 36-55th, the 32-53rd, the 36-53rd, or the 36-57th nucleotides. Since all other claims of this application and the application 1 cite Claim 1, also in the claim of others [application / 1 / this application and], invention is different.

Therefore, Reason 1 eliminated.

(3-2) Claim 1 of Reason 2 this application is as follows. "It is antisense oligomer which makes possible skipping of the 53rd exon of the Homo sapiens dystrophin gene, It consists of a base sequence complementary to the arrangement which consists of the 36-60th nucleotides from the five prime end of the 53rd exon of the Homo sapiens dystrophin gene, Antisense oligomer. Below " calls the oligomer which starts Claim 1 for simplification "oligomer of the present invention", and it calls the arrangement of oligomer of the present invention "the 36-60th." The present invention is invented for the purpose of solving the problem (paragraph 0011 of Description of this application) that the antisense oligomer which derives skipping of the exon 53 of a dystrophin gene strongly is provided.

On the other hand, in Cited Document 2, [five prime end / of the 53rd exon of the Homo sapiens dystrophin gene] [antisense oligomer which consists of the base sequence of arrangement complementary to any one which consists of the 38-62nd (h53AON1) and the 23-47th nucleotides (H53A (+23+47))] It describes (Example 3).

If the present invention and an invention in the cited document 2 are contrasted, both are different in that the oligomer in which oligomer of the present invention has the arrangement concerned in Cited Document 2 although arrangement is specified by the 36-60th is not described. The composition which relates to below in the different feature concerned explains not easily conceiving and producing the effect outstanding by composition which requires the present invention for the different feature concerned.

(i) In Example 3 of easily-conceiving-composition concerning the different feature Cited document 2, the skipping activity of the exon 53 is verified, and before long, oligomer of SEQ ID NO:429 is described as having urged exon 53 skipping the most efficient as shown in Fig.4 B-F. And SEQ ID NO: Oligomer of 429 is described as having the same activity as H53A (+23+47) (SEQ ID NO:609) was proved. This description shows that H53A (+23+47) (SEQ ID NO: 609) has the highest skipping activity (activity equivalent at least). They are H53A (+23+47) (SEQ ID NO: 609) and SEQ ID NO also from Fig.4 H (the following figure) : While only oligomer of 429 is deriving skipping efficiently, It turns out that other oligomer (H53A (+39+69), H53AON1, H53A (-12+10), 008) is inferior in skipping activity, and skipping has hardly arisen.

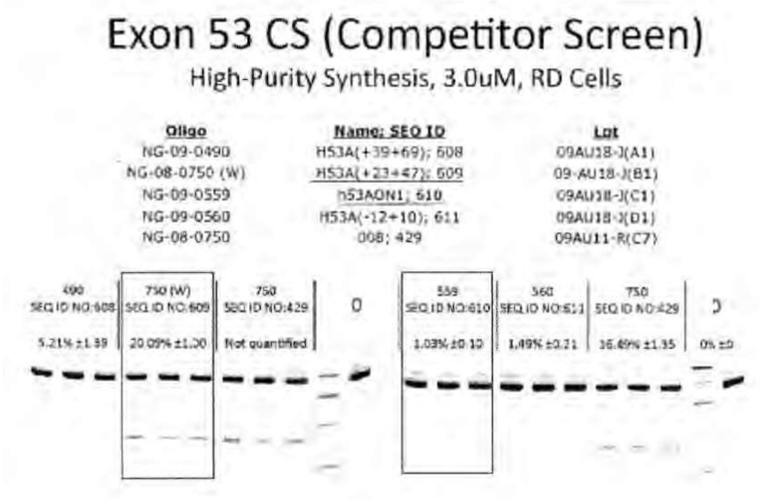
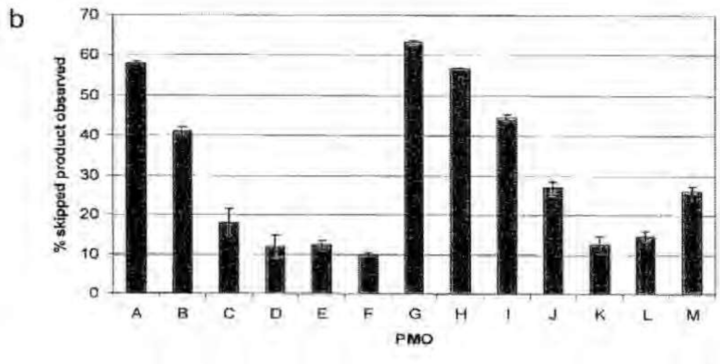


FIG. 4H

Therefore, when it was a person skilled in the art aiming at solution of the problem given to the present invention, and the description of Cited document 2 was touched, naturally its attention must have been paid to H53A (+23+47). And in order to obtain the antisense oligomer which derives skipping strongly, producing the oligomer which carries out the target of the 23rd-47th arrangement or its periphery must have been motivated. Although it is h53AON1 (the 38-62nd) that has the arrangement near oligomer (the 36-60th) of the present invention among the oligomer disclosed in Cited document 2 on the other hand, even if this looks at the description of Example 3 of Cited document 2, and the data of Fig.4 H, it is clear that its skipping efficiency is low. From this point, neither the 38th-62nd arrangement nor its periphery charmed the person skilled in the art's interest as a target of skipping.

Therefore, if comprehensive determination of the description of Cited document 2 is carried out, it turns out that there is cross motion clear about a person skilled in the art thinking out the composition concerning the different feature in Cited Document 2.

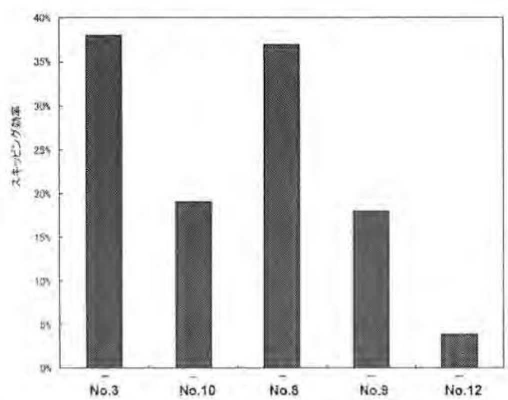
Next, when the description of Cited document 3 is verified, it is shown that PMO-G (the 30-59th) has the highest activity in Cited document 3, on the other hand, it is shown that PMO-A (the 35-59th: a base sequence from the arrangement of oligomer of the present invention 1 base ***) which has the arrangement near oligomer (the 36-60th) of the present invention has activity lower than PMO-G (Fig.8 b: the following figure). So, the person skilled in the art who touched the description of Cited document 3 can say it that it has motivated to carry out the target of the 30-59th in quest of high activity like the case of Cited document 2.



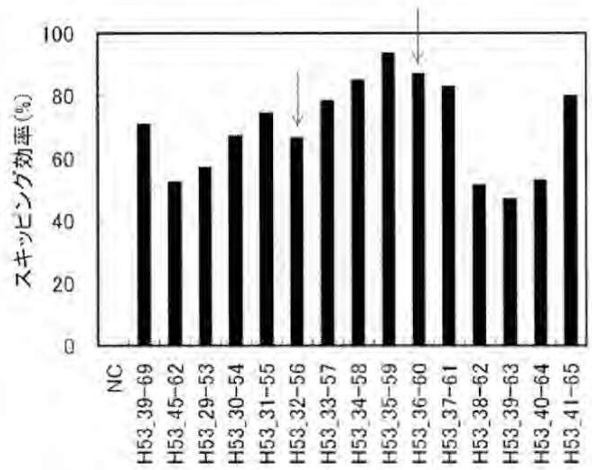
Therefore, even if a person skilled in the art touches Cited document 2 and the description of 3, he should not have it motivated to search for the target arrangement of oligomer of the present invention, and cannot easily conceive the composition which relates to the different feature as the result.

As mentioned above, since the composition concerning the different feature is not easily conceived from the description of a cited document, the present invention has an Inventive Step.

(ii) It is checked by the experiment that oligomer of the producing-effect outstanding by composition which requires the present invention for the different feature concerned present invention has the skipping activity which was excellent in Cited document 2 and 3 as compared with oligomer of a description. SEQ ID NO made the highest [skipping activity] in Cited document 2 : 429 oligomer, PMO-G (the 30-59th) made the highest [skipping activity] by Cited document 3 is produced as PMO No. 16 and PMO No. 12 in working example in this application, respectively. Comparison with oligomer of Cited document 3 is explained first. In the first place, it **s from the Fig.3 of this application that PMO No. 3 (the 32-56th) are highly active than PMO No. 12. [Fig. 3]

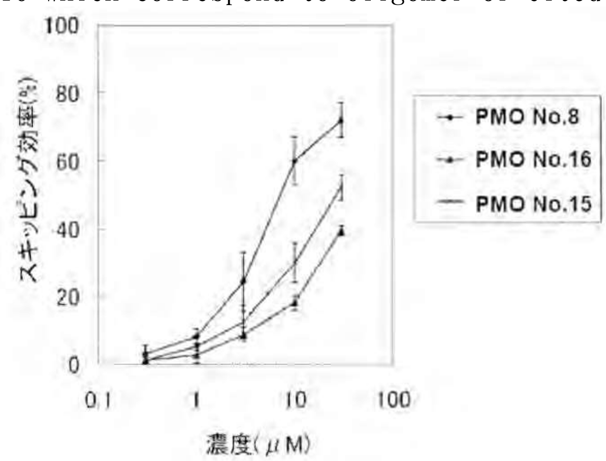


Next, if the Fig.16 of this application is referred to, it turns out that H53_36-60 which corresponds to oligomer of the present invention has activity higher than H53_32-56 which corresponds to PMO No.3. [Fig. 16]



[therefore height with active PMO-G (the 30-59th) of oligomer of the present invention, PMO NO. 3 and Cited document 3] It is "PMO-G of the oligomer >PMO No. 3> Cited document 3 of the present invention", and it turns out that oligomer of the present invention is highly active than PMO-G of Cited document 3.

On the other hand, about comparison with oligomer of Cited document 2, this application Fig.18 shows that PMO No. 8 (the 36-56th) are highly active than PMO No. 16 which correspond to oligomer of Cited document 2. [Fig. 18]



Next, it turns out that Fig.2 -4 to PMO No. 8 have activity comparable as PMO No. 3. Since it is highly active than PMO No. 3, as stated in the top, [oligomer of the present invention] H53A (+23+47) (PMO No. 16) of oligomer of the present invention, PMO No. 8, PMO NO. 3, and Cited document 2, [active height] It is "H53A (+23+47) of the oligomer >PMO No. 3 nearly-equal-toPMO No. 8> Cited document 2 of the present invention", and it turns out that oligomer of the present invention is highly active than H53A (+23+47) of Cited document 2.

[Cited document 2 and oligomer with the highest skipping activity at 3] Since it is shown that the target arrangement (the 36-60th) of oligomer of the present invention carries out the target of the different region, it can be said that having the activity with which oligomer of the present invention exceeds Cited document 2 and oligomer of 3 is the effect which could not predict from the description of these document and was excellent.

As mentioned above, since oligomer of the present invention produces the effect where a person skilled in the art could not predict and which was excellent in him from the description of the cited document, it has an Inventive Step also from the surface of an effect.

(iii) The composition cannot think out oligomer of the present invention easily from the description of a cited document, and since it produces the effect which could not predict and was excellent, it has an Inventive Step, as stated on the Summary. Since all Claims 2-10 cite Claim 1, invention concerning these claims has an Inventive Step similarly.

4. Since I consider that all reasons for refusal eliminated from the thing more than a conclusion, I ask you to give me the decision of the purport that it should patent, after a re-degree examination per this application.

End of document

特許査定

特許出願の番号	特願２０１５－２５６９６２
起案日	平成２９年　６月２９日
特許庁審査官	福澤　洋光　　　　　　　　３９６３　４Ｂ００
発明の名称	アンチセンス核酸
請求項の数	１０
特許出願人	日本新薬株式会社（外　１名）
代理人	小林　浩（外　３名）

この出願については、拒絶の理由を発見しないから、特許査定をします。

部長／代理	審査長／代理	審査官	審査官補	分類確定官
	松波　由美子	福澤　洋光		太田　雄三
	９２６１	３９６３		３９５９

１．出願種別	分割
２．参考文献	有
３．特許法第３０条適用	無
４．発明の名称の変更	無

５．国際特許分類（ＩＰＣ）

Ｃ１２Ｎ	１５／００	ＺＮＡＧ,
Ａ６１Ｐ	２１／０４	,
Ａ６１Ｋ	４８／００	,
Ａ６１Ｋ	３１／７０８８	,
Ａ６１Ｋ	３１／７１２	,
Ａ６１Ｋ	３１／７１２５	

６．菌寄託

７．出願日の遡及を認めない旨の表示

参考情報

特許出願の番号　　　　　　特願２０１５－２５６９６２

１．調査した分野（ＩＰＣ，ＤＢ名）

Ｃ１２Ｎ　　１／００－１５／９０
ＣＡ／ＭＥＤＬＩＮＥ／ＢＩＯＳＩＳ／ＷＰＩＤＳ（ＳＴＮ）
ＪＳＴＰｌｕｓ／ＪＭＥＤＰｌｕｓ／ＪＳＴ７５８０（ＪＤｒｅａｍＩＩＩ）
Ｇｅｎｂａｎｋ／ＥＭＢＬ／ＤＤＢＪ／ＧｅｎｅＳｅｑ
ＰｕｂＭｅｄ

２．参考特許文献

特開２０１４－０５４２５０　　（ＪＰ，Ａ）
国際公開第２０１０／０４８５８６　　（ＷＯ，Ａ１）
米国特許出願公開第２０１０／０１６８２１２　　（ＵＳ，Ａ１）
特開２００２－０１０７９０　　（ＪＰ，Ａ）
国際公開第２００８／０３６１２７　　（ＷＯ，Ａ１）

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Decision to Grant a Patent

Application number: Japanese Patent Application No. 2015-256962
Date of Drafting: Heisei 29(2017) June 29
Patent examiner: FUKUZAWA, Hiromitsu 3963 Four B00
Title of the invention:ANTISENSE NUCLEIC ACIDS
The number of claims: 10
Applicant: Nippon Shinyaku Co.,Ltd. (and 1 others)
Representative: KOBAYASHI, Hiroshi (and 3 others)

This application is to be granted a patent, since no reason for refusal has been found.

A Director General(p.p.), a Director(p.p.), an examiner, assistant examiner A Manager for Determination of Classification, MATSUNAMI, Yumiko, FUKUZAWA, Hiromitsu, and OTA, Yuzo 9261 3963, 3959

1. Distinction of Patent: Division
2. Reference: see below
3. Application of Patent Act Article 30: not applicable
4. Change of the Title of the Invention: No

5. International Patent Classification (IPC)

C12N 15/00 ZNAG, A61P 21/04 , A61K 48/00 , A61K 31/7088 , A61K 31/712 , A61K 31/7125

6. Deposit of Microorganisms

7. Indication of Unacceptance of Retroactivity to the Filling Date

Reference information

Application number: Japanese Patent Application No. 2015-256962

1. Technical Fields Searched (IPC, DB Name)

C12N 1/00-15/90

CA/MEDLINE/BIOSIS/WPIDS(STN)JSTPlus/JMEDPlus/JST7580(JDreamIII)Genbank/EMBL/DDBJ/GeneSeqPubMed

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United States patent publication of unexamined application the 2010/0168212 (US, A1)
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International-Publication the 2008/036127 (WO, A1)

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POPPLEWELL and L.J. et al., Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene., Mol. Ther., 2009, and Vol.17 No.3 and p.554-561POPPLEWELL, L.J. etal., Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials., Neuromuscul. Disord., February, 2010, and Vol.20 No.2 and p.102-110 AARTSMA-RUS, A. et al., and Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy., Neuromuscul. Disord., 2002, Vol.12 Supplement, p.S71-S77WILTON, S.D. et al., Mol. Ther., and Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript., 2007, and Vol.15 No.7, p.1288-1296